PCU52918

MRS. LESLEY GORONCY
46 CAMBAGE STREET
PINDIMAR NSW 2324

24 April 2014

Attention: Director, Industry, Key Sites and Social Projects
Development Assessment Systems & Approvals
Planning & Infrastructure
GPO Box 39
SYDNEY NSW 2001

Dear Ms. Goward:

Application Name: Proposed Pindimar Abalone Farm
Application #: MP 10_0006

I object to the proposed Pindimar Abalone Farm.

Declaration re: political donations: I declare that I have not made any political donations in the previous two years.

I make this submission as a result of reading a copy of the report "Investigating and Managing the Perkinsus-related mortality of blacklip abalone in NSW". Authors G. W. Liggins and J. Upston. FRDC Project No. 2004/084, June 2010. Industry and Investment NSW, Fisheries Final Report Series No. 120 ISSN 1837-2112. Authorised by the NSW Government Industry & Investment and Australian Government Fisheries Research and Development Corporation. This report is attached.

I would like to draw your attention to:

a) Multiple Strains of the Perkinsus Disease: pages 47, 61, 70, 74, 76 and 91.
b) Environmental Factors: page 80
c) Temperature: pages 92, 93, 94, 108, 110, 112, 120, 122, 123, 124, 132, 137, 149
d) Precautionary Approach: page 132

The proponents should not be allowed to industrialise Port Stephens.

Yours sincerely,

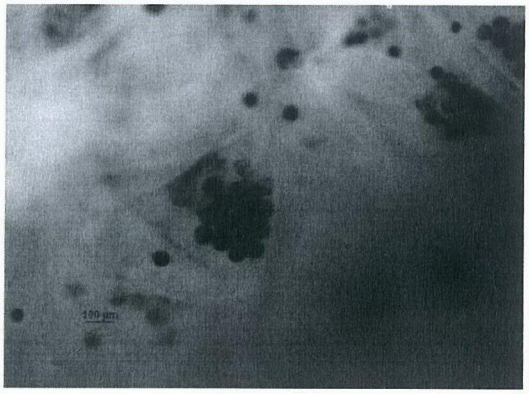
L. Goroncy

LESLEY GORONCY

Investigating and managing the *Perkinsus*-related mortality of blacklip abalone in NSW

G. W. Liggins and J. Upston

Industry & Investment NSW
Cronulla Fisheries Research Centre of Excellence
PO Box 21, Cronulla, NSW, 2230
Australia



FRDC Project No. 2004/084

June 2010

Industry & Investment NSW –
Fisheries Final Report Series
No. 120
ISSN 1837-2112

NSW GOVERNMENT | Industry & Investment

Australian Government
Fisheries Research and Development Corporation

Investigating and managing the *Perkinsus*-related mortality of blacklip abalone in NSW

June 2010

Authors:	Liggins G.W. and Upston J.
Published By:	Industry & Investment NSW (now incorporating NSW Department of Primary Industries)
Postal Address:	Cronulla Fisheries Research Centre of Excellence, PO Box 21, Cronulla, NSW, 2230
Internet:	www.industry.nsw.gov.au

ISSN 1837-2112

Note: Prior to July 2004, this report series was published by NSW Fisheries as the 'NSW Fisheries Final Report Series' with ISSN number 1440-3544. Then, following the formation of the NSW Department of Primary Industries the report series was published as the 'NSW Department of Primary Industries – Fisheries Final Report Series' with ISSN number 1449-9967. The report series is now published by Industry & Investment NSW as the 'Industry & Investment NSW – Fisheries Final Report Series' with ISSN number 1837-2112.

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ACKNOWLEDGEMENTS

A considerable number of people provided advice and assistance to us during the course of this project.

Pascal Geraghty was employed as a casual technician on this project and assisted with fieldwork and processing samples for Ray's test. We also thank the other technical and scientific staff from the Cronulla Fisheries Centre of Excellence (I&I NSW) who assisted with fieldwork and laboratory work from time to time.

Dr Craig Blount (*The Ecology Lab Pty Ltd)* was contracted to provide diving services to assist project staff during the broad-scale survey.

Shareholders and divers in the NSW abalone fishery including Steve Bunney, Darren Clark, Mick Herrman, Paul Staight, Ari Miinin and Troy Miller collected samples for us under section 37 permits. We also thank the commercial and recreational abalone divers who participated in interviews and the abalone processors who also provided information.

Members of the Abalone Management Advisory Committee, Dr Duncan Worthington and abalone shareholders/divers John Smythe and the late Chris Grover provided invaluable advice and discussion, particularly during project development.

Additional historical information about the abalone fishery in NSW was provided by Dr Gary Hamer and Laurie Derwent.

Jane Cameron and Bill Booth (UTS Dept of Medical and Molecular Biosciences) provided laboratory facilities and equipment including an autoclave that we used to make up the thioglycollate media for Ray's test.

Dr Paul Gill, assisted by technician Nick Stevens, (NSW Regional Veterinary Laboratory, Wollongbar, now within I&I NSW) performed the processing of tissue samples and histological examinations.

Maria Hardy, Janet Eamens, Mark Turner and Louise Brown (EMAI, Diagnostic and Analytic Lab.) and Narelle Richardson (UTS Dept of Environmental Sciences) provided low-temperature freezer storage for tissue samples, necessary prior to PCR assays. Graeme Fraser (Wollongbar Agricultural Institute) assisted us with long-term storage of abalone samples prior to and since histological examination.

Dr Craig Hayward (SARDI Aquatic Sciences), Dr Robert Lester (Dept. of Microbiology and Parasitology, UQ), Dr Judith Handlinger and Richmond Loh (Animal Health Laboratories (DPIW Tasmania), Dr Chris Dungan (Maryland DNR Cooperative Oxford Laboratory, USA) provided advice about Ray's test and associated sampling techniques.

Assoc. Prof. Kimberly Reece (School of Marine Science, Virginia Institute of Marine Science, USA) and staff at her laboratory performed the PCR assays and gene sequencing and provided assistance in interpretation of results from these tests. Dr Serge Corbeil (CSIRO Aquatic Animal Health Laboratory) provided initial advice and also ongoing discussion and advice about PCR diagnostic methods and interpretation of results. Dr Leslie Reddacliff and Ian Marsh (EMAI, I&I NSW) performed pilot PCR's for this project.

Dr Des Beechey (Australian Museum) confirmed the identification of mollusc species that were collected during the survey of alternative mollusc hosts.

Consultation and advice regarding aspects of parasitology and epizootiology were provided by Prof. John Ellis (Dept. of Medical and Molecular Biosciences, UTS), Dr Dick Callinan and Matt Landos (previously at Wollongbar Agricultural Institute / NSW DPI Regional Veterinary Laboratory), the late Dr Chris Baldock and Dr David Kennedy (AusVet Animal Health Services Pty Ltd) and Dr Fran Stephens (Dept. of Fisheries, WA).

Water quality and hydrology data were provided by DR Peter Tate and Dr Marcus Scammel (Sydney Water Corporation), Dennis Day and Barbara McRae (Gosford City Council) and Mark Gebhard, Ron Grudoff and Darren Cleary (Hunter Water Corporation).

Dr Doug Ferrell, Jeffrey Go and Jane Frances (I&I NSW) and David Kennedy (AusVet Animal Health Services Pty Ltd) reviewed and provided valuable comments on the report.

NON-TECHNICAL SUMMARY

2004/084	Investigating and managing the *Perkinsus*-related mortality of blacklip abalone in NSW.

PRINCIPAL INVESTIGATORS: G.W. Liggins

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OBJECTIVES:

(1) Compile and document the historical evidence about the spread of *Perkinsus*-related mortality of abalone in NSW.

(2) Describe the pathogenesis and make initial investigations of the epizootiology of the mortality of abalone with particular reference to the role of *Perkinsus*.

(3) Contribute to the development of strategies to manage populations of abalone that have, or might be, affected by *Perkinsus*-related mortality and, in particular, evaluate the need for a second phase of research.

NON TECHNICAL SUMMARY:

Since the early 1990's, a significant proportion of blacklip abalone (*Haliotis rubra*) along approximately 500 km of the NSW coastline between Port Stephens and Jervis Bay have died. Sporadic histological examination of moribund abalone since 1992 and a survey of infection prevalence in abalone using Ray's test in 2002 confirmed infections of a protistan parasite, *Perkinsus* sp., in abalone. It has been assumed that the species is *P. olseni*, but this has not been confirmed (prior to this project). It has been unclear to what extent mortality of abalone has been caused by *Perkinsus*. Other factors may also have been responsible for the mortalities. Should the *Perkinsus*-related mortality spread further south into increasingly high density stocks of abalone, there would be a rapid escalation of impacts.

Against this background, there was a need to: document historical evidence about the spread of *Perkinsus*-related mortality of abalone in NSW; describe the pathogenesis and epizootiology associated with the mortality of abalone and the role of *Perkinsus*; and to contribute to the development of management strategies to manage populations of abalone that have, or might be affected by *Perkinsus*-related mortality and evaluate the need for further research.

Based on structured interviews of divers, mass mortalities of abalone occurred during the year (+/- 1 year): 1992 on the Central Coast of NSW, 1993 at Sydney-Wollongong, 1996 at Kiama and 2000 at Port Stephens. A sudden decline in abundance of abalone, unaccompanied by observations of morbidities or mortalities, occurred at Jervis Bay during 2000 – 2002.

Indices of abundance of abalone were derived from fishery-dependent catch and effort data, fishery-independent surveys and estimates provided by interviewed divers. Stocks of abalone between Port Stephens and Jervis Bay were significantly depleted by at least 63%, due to fishing mortality, prior to the documented mortality events in the 1990's. Stocks were further depleted by an estimated 74% due to a combination of the documented mass mortality events during the 1990's and limited fishing. These estimates suggest that the stock of abalone between Port Stephens and

Jervis Bay may have been depleted to less than 10% of virgin abundance. Given limited larval dispersal and localised recruitment in abalone populations, the recovery of stocks of blacklip abalone between Port Stephens and Jervis Bay is likely to be a slow process.

A broad-scale survey of the prevalence and intensity of *Perkinsus* infections in abalone in 2005 involved the collection of 1190 abalone from 59 sites within 12 locations along the NSW coast. Three diagnostic methods were used to identify infections of *Perkinsus*: Ray's test on samples of gill tissue from all abalone sampled; histology on multiple organs/tissues on a subset of abalone; and polymerase chain reaction (PCR) assays and subsequent gene sequencing on gill and mantle tissue from a subsample of abalone.

Ray's test was the most sensitive diagnostic method for the detection of *Perkinsus*. Histology detected only 33% of the infections and PCR detected only 54% of the infections detected by Ray's test. *P. olseni* was positively identified, by PCR and gene sequencing, as a *Perkinsus* species infecting blacklip abalone in NSW. Identification, by PCR and gene sequencing, of a *Perkinsus* sp. with a previously unknown ITS-region gene sequence (referred to as *Perkinsus* sp.-variant ITS) suggests the presence of a variant strain of *P. olseni,* or alternatively, a previously undescribed *Perkinsus* species.

In 2005, *Perkinsus* sp. infections in abalone were identified at multiple survey sites, within multiple survey locations between Point Perpendicular (near Laurieton) on the north coast of NSW) and St. Georges Head (near Jervis Bay) on the south coast of NSW. *Perkinsus* sp. was also identified at a single isolated site (Merimbula) on the far south coast of NSW. Detection of *Perkinsus* sp. infections in abalone during 2005, at locations to the north and south of the previously documented range, represents an extension of the documented geographic range of *Perkinsus* sp. infections in blacklip abalone in NSW. Actual extension of geographic range since 2002 cannot be concluded because the northern and southern boundaries of distribution identified in 2005 were outside the geographic range of the 2002 survey. Within the documented geographic range of *Perkinsus* sp., *P. olseni* was identified by PCR between Port Perpendicular (within location "North Coast") and St. Georges Head (within location "South Jervis Bay"). *Perkinsus* sp.-variant ITS was identified by PCR between Crowdy Head North (within location "Crowdy Head") and St. Georges Head (within location "South Jervis Bay") and on the far south coast at Merimbula (within location "Tathra / Eden").

Prevalence of *Perkinsus* sp. infections in abalone in 2005 differed significantly among locations and among sites within locations. Prevalence was greatest in populations of abalone at the northern and southern margins of the infected zone between locations North Coast and South Jervis Bay (excluding the "outpost" of infection at Merimbula). This was consistent with the pattern of prevalence found in 2002, when maximum prevalence occurring at the northern (Port Stephens) and southern (South Jervis Bay) boundaries of the infected zone.

Intensity of *Perkinsus* sp. infections in abalone (based on Ray's test grade) differed significantly among locations in 2005. Although there was no significant difference in the prevalence of *Perkinsus* sp. infections in abalone in 2005 compared to 2002, intensity of infections was significantly greater in 2002 compared to 2005. The greater intensity of P*erkinsus* sp. infections in 2002 compared to 2005 explains the better relative sensitivity of Ray's test and histology in 2002 compared to 2005. This is because the relative sensitivity of these diagnostic methods is better for high-grade infections than low-grade infections.

Infections of *Perkinsus* sp. in abalone, identified by histology, were generally systemic with intestine, gill, kidney, stomach and heart most frequently infected. Less frequently infected were epipodium, the digestive gland, oesophagus, muscle and haemolymph within the hemocoel. These observations were consistent with findings from a previous study on samples from a survey in 2002-03.

Substantial tissue and organ damage occurred in abalone with high-intensity infections. Necrotic tissue associated with *Perkinsus* sp. trophozoites and tomonts was observed in samples from 2002-03 and 2005. In the most severe infections (2002-03), disruption of the gut epithelium and infarction in the gills, suggested impairment to normal nutrient absorption and respiration. Infiltration and aggregation of haemocytes provided evidence of host response and there was a positive relationship between infection intensity (Ray's test grade) and haemocyte activity. The evidence of substantial tissue necrosis, organ damage and haemocyte activity associated with *Perkinsus* sp. cells in samples from both 2002-03 and 2005 provides solid evidence that *Perkinsus* sp. is pathogenic to blacklip abalone in NSW. More specifically, pathology associated with the 5 specimens from 2005, within which *P. olseni* was positively identified by PCR, provides solid evidence of *P. olseni* as a pathogen in blacklip abalone in NSW. Because there was no observation of *Perkinsus* by histology for any of the 5 abalone identified by PCR and gene sequencing as having *Perkinsus* sp.-variant ITS infections, the pathogenicity of *Perkinsus* sp.-variant ITS remains unresolved.

Infection intensity did not affect the length-weight condition of abalone sampled in 2005. Because extremely high-grade infections of *Perkinsus* sp. (Ray's grade ≥ 4) were absent from samples in 2005, this conclusion is restricted to abalone with lower infection intensity (i.e., $\leq$ Ray's grade 3). The lack of encapsulation of *Perkinsus* sp. cells by haemocytes, as described for *P. olseni* infections in South Australian blacklip abalone, suggests a difference in the virulence of *P. olseni* (or strain of *P. olseni* or *Perkinsus* sp.) and/or a difference in the resistance of blacklip abalone in NSW compared to South Australia.

No pathogens other than *Perkinsus* sp. were identified by histology in tissue samples from the 2005 survey. Nor were any other pathogens identified in samples from the 2002-03 survey.

The importance of population variables including gender and size on prevalence and intensity of infections was examined. Prevalence of *Perkinsus* sp. infections was gender-specific with infections more frequent in female (10.9%) than in male (5.0%) abalone in 2005. There was no gender-specific difference in infection intensity. Prevalence and intensity of *Perkinsus* sp. infections in abalone during 2005 was not dependent on the size (length) of abalone.

A small-scale survey to identify *Perkinsus* sp. infections in alternative mollusc hosts, at sites where *Perkinsus* sp. was prevalent in abalone (in the vicinity of Jervis Bay), did not identify any alternative host species.

Investigations were made of the likelihood of environmental variables (acting alone or as auxiliary factors operating with *Perkinsus* sp.) as the cause of documented mortality events. There were no environmental factors that were consistently associated by interviewed divers with mortality events that were consistent across the documented mortality events. The potential to examine associations between water quality and observed mortality events was limited by the spatial and temporal scales at which such data was available. The documented mortality events at specific locations in specific years were not consistently associated with increases, decreases, maximum or minimum values in any of the indices of water temperature examined. Mortality events were, however, associated with indices of water temperature in excess of 20°C and the greater intensity of infections identified in 2002 compared to 2005 corresponded with greater water temperature in 2002 compared to 2005.

Based on the consistency of findings from this project and from other studies in Australia and internationally, with hypotheses concerning factors involved in the cause of documented mortality events, the likelihood of alternative hypotheses of cause being correct was graded "low", "medium" or "high". It was concluded highly likely that *Perkinsus* sp. was a necessary factor for the documented mortality events. The likelihood that water temperature (above some threshold) was an additional component of cause was also considered high. Whether or not another

environmental factor(s) was also a component of cause was indeterminate. It was also considered likely, based on available evidence, that *Perkinsus* sp. has not been endemic and broadly distributed along the NSW coast for many years prior to the documented mortality events. The spatial and temporal pattern of documented mortalities and finding that *Perkinsus* sp. infections were greatest at the boundaries of the infected zone were consistent with a progression of *Perkinsus* sp. through naïve populations of abalone.

Transmission of *Perkinsus* sp. within and among populations of abalone at locations along the NSW coast may have resulted from passive or active mechanisms. Multiple fishing-related activities were identified that could have translocated abalone infected with *Perkinsus* sp. and facilitated active transmission of the parasite into populations of abalone that were disease-free. Activities that involved translocations at spatial scales of less than 100's of km included: harvesting of abalone by divers at individual drops; hanging of abalone over the side of the boat or pumping water over abalone in the boat during a day's fishing; hanging of catch during multi-day trips; and shucking of abalone and discard of viscera. Activities that involved translocation of abalone at greater spatial scales included: movement of divers, boats and equipment between locations; movement of catches to processors and distributors; and the distribution of abalone viscera as bait.

In addition to these possible mechanisms for active transmission, experiments involving the translocation of abalone from the wild to a breeding facility and the subsequent translocation of hatchery-reared juveniles and larvae from the facility to the wild between 1999 and 2001 could have inadvertently translocated abalone carrying *Perkinsus* sp. infections.

Whereas the spatial and temporal pattern of documented mass mortality events and of *Perkinsus* sp. infections between the north coast of NSW and South Jervis Bay was consistent with the passive transmission of *Perkinsus* sp. among adjacent populations of abalone, possibly supplemented by active mechanisms of transmission, the isolated "outpost" of *Perkinsus* sp. infection in abalone at Merimbula is likely to have resulted from an active mechanism of transmission. The mechanism could have involved a mobile marine species or bird acting as a vector, the fishing-related activities that involved translocation of abalone or abalone viscera over 100's of km, or perhaps the translocation/reseeding experiments that included the release, in 2001, of hatchery-reared juvenile abalone at Tura Pt (5 km to the north of the Merimbula site at which *Perkinsus* sp. was identified in 2005).

A precautionary approach to the management of populations of abalone affected by *Perkinsus* sp. in NSW is warranted. Whilst this project has made significant inroads into understanding the pathogenesis and epizootiology of perkinsosis and the documented mortality of abalone during the 1990's, understanding of the epizootiolgy of perkinsosis in NSW and in other Australian jurisdictions is incomplete. There is potential for further impact of the disease on the commercial and recreational fishery concentrated on the south coast of NSW.

Closures to fishing in areas where *Perkinsus* sp. is known to be present, accompanied by closures in buffer zones between infected areas and disease-free areas is one option for limiting the possibility of transmitting *Perkinsus* sp. from areas where the parasite is known to occur into disease-free areas. This would involve, in NSW, an extension of the existing closure between Port Stephens and Jervis Bay to the north coast of NSW, a buffer zone implemented south of St. Georges Head (South Jervis Bay) and another closure, including buffer zones, implemented in the vicinity of Merimbula.

Another approach to minimising the risk of *Perkinsus* sp. transmission concerns modification of existing fishing practices that involve a risk of transmission. The development of "Standard Operating Procedures" for the decontamination of equipment (tools, boats, people and clothing, transport containers) provides a means of minimising this risk of translocating viable cells of

Perkinsus sp. resulting from the movement of divers, boats and equipment between locations. Standard operating procedures already exist as a voluntary code of conduct. The level of compliance with this code of conduct is unknown and the option of legally requiring and enforcing standard operating procedures needs to be evaluated.

In the absence of closures preventing the harvesting of abalone potentially infected with *Perkinsus* sp., it is inevitable that abalone infected with *Perkinsus* sp. will be received by abalone processors. A review of current procedures used by processors and audit against best practice procedures is recommended.

To counter the risk of transmitting *Perkinsus* sp. (and abalone viral ganglioneuritis), the distribution and use of abalone viscera as bait or burley was made illegal in NSW in December 2007.

Given the absence of fishery-dependent data since the closures to commercial fishing between Port Stephens and Jervis Bay and limited fishery-independent survey sites, assessment of the current status of the severely depleted abalone stocks in this region is problematic. A structured fishing survey, done in 2007, involved a limited number of commercial abalone divers fishing at specified drops (specific locations) between Port Stephens and Jervis Bay. The specified drops at which fishing occurred were selected from "historically productive drops" identified by the commercial abalone divers interviewed during this project. Continued monitoring of abundances of abalone at fishery-independent survey sites within this region also provides a means of monitoring abundance and recovery of stocks in this region of the fishery and will inform management decisions concerning access of fishers to the resource. Whether abalone stocks in *Perkinsus* sp.-affected areas of Region 1 can sustain commercial harvesting has not yet been determined. In addition to controlling risks of transmission of *Perkinsus* sp., a future harvest strategy for this region will need to recognise the possibility that there may be future mortality events due to *Perkinsus* sp. in addition to mortality from future fishing.

Given the incomplete understanding of the epizootiology of perkinsosis and associated mortality of abalone in NSW, priorities for future monitoring and research were identified. Future repeats of the broad-scale survey completed in 2005 would facilitate monitoring of any future expansion in the geographic range of *Perkinsus* sp. infections in abalone. Monitoring and associated sampling of future mortality events is also fundamental to furthering understanding of epizootiology. Surveys, supplementary to repeats of the broad-scale survey, could be done to examine intra-annual and inter-annual variations in prevalence and intensity of *Perkinsus* sp. infections. As a component of future broad-scale surveys, application of a suitably sensitive testing methodology for the identification of *P. olseni* and *Perkinsus* sp.-variant ITS (ideally real-time PCR), across multiple organs would provide further evidence for the presence/absence of *Perkinsus* sp. infections in abalone sampled from sections of the NSW coast for which infections were not detected during the 2005 survey. Sampling of mollusc species other than abalone and subsequent testing for the presence of *Perkinsus* sp. infections, in conjunction with a repeat of the broad-scale survey, would directly address the uncertainty associated with the identification of alternative hosts.

As an adjunct to the next repeat of the broad-scale survey, or as a stand-alone survey, identification of the existence of *Perkinsus* sp. infections at survey sites where juvenile abalone were released on the south coast of NSW during the reseeding experiments during 1999 – 2001 and at control sites (where reseeding did not occur) would provide a test of the hypothesis that *Perkinsus* sp. infections exist in abalone at sites in close proximity to reseeding sites but do not exist at sites away from reseeding sites on the south coast of NSW.

Uncertainty about sufficiency of the presence of *Perkinsus* sp. and water temperature elevated above some threshold as the cause of the development of perkinsosis and subsequent mortality of

abalone can be further investigated in several ways. In particular, there is a need for controlled laboratory-based experiments concerning the role of water temperature in disease progression.

Development and establishment, in Australia, of real-time PCR assays for the diagnosis of *P. olseni* and *Perkinsus* sp.-variant ITS infections in tissue samples is recommended and underpins the fast and efficient identification of *Perkinsus* in samples from future surveys and experiments. Clarification of the genetic and taxonomic status and geographic distribution of *Perkinsus* sp.-variant ITS is also required.

Outcomes Achieved

All objectives of this project have been achieved.

Historical evidence about mass mortalities of abalone in NSW was collected through structured interviews with divers and the timing and locations of these events was documented. Based on information from interviews with divers, fishery-dependent catch and effort data from the fishery and fishery-independent survey data, the depletion of abalone stocks prior to and since documented mortality events was estimated.

A broad-scale survey of the prevalence and intensity of *Perkinsus* infections in abalone and related analyses and conclusions concerning the geographic distribution of *Perkinsus* sp., *P. olseni* and variant strain or species (*Perkinsus* sp.-variant ITS), patterns of prevalence and intensity of infections among locations and among sites within locations and underpinned analyses concerning the pathogenesis and epizootiology associated with documented mortalities and perkinsosis.

Information about the pathogenesis of *Perkinsus* sp. and *P. olseni* infections in abalone came from examination of the histology associated with a subset of samples from the broad-scale survey. Findings including the systemic nature of infections associated with substantial tissue necrosis, organ damage and haemocyte activity provided direct evidence that *Perkinsus* sp. and specifically, *P. olseni*, is pathogenic to blacklip abalone in NSW.

Investigations of the epizootiology of perkinsosis and associated mortalities of abalone included: investigation of infection prevalence and intensity related to population variables; a small-scale survey to identify alternative host species; the likelihood of environmental variables contributing to the cause of documented mortalities; analyses of the consistency of available evidence with alternative hypotheses concerning factors involved in the cause of documented mortalities; the consistency of available evidence with alternative hypotheses concerning the historical distribution of *Perkinsus* sp. along the NSW coast; and identification of mechanisms of transmission (passive and active) that may have been involved in the spread of perkinsosis and associated mortalities.

Based on current knowledge of the geographic distribution of *Perkinsus* sp., pathogenesis and epizootiology of *Perkinsus* sp. in NSW and findings from other studies in Australia and internationally, options of management of the population of abalone and fisheries in NSW were reviewed. To gain further understanding of the epizootiology of perkinsosis and related mortalities and inform management in the future, multiple high-priority monitoring and research programs were recommended.

KEYWORDS:

Perkinsus sp., *Perkinsus olseni*, perkinsosis, Ray's test, histology, PCR, pathogenesis, epizootiology

1. BACKGROUND AND NEED

Since the early 1990's, a significant proportion of the abalone along approximately 500 km of the NSW coastline have died. The mortality apparently commenced around Sydney and the Central Coast in the early 1990s, and gradually spread north to Port Stephens and south to Jervis Bay. Sporadic histological examination of moribund abalone since 1992 and a survey of infection prevalence in blacklip abalone (*Haliotis rubra*) using Ray's test in 2002 confirmed infections of a protistan parasite, *Perkinsus* sp., in abalone. It has been assumed previously, that the species is *P. olseni*, but this has not been confirmed. Mortality of abalone has affected reefs that historically produced the order of 80 t of abalone, valued at approximately $3 million per year. It is unclear whether the mortality is continuing to spread. Should the mortality continue to spread further south into increasingly high density stocks of abalone, there will be a rapid escalation of impacts.

It is unclear to what extent the spread of mortality of abalone has been caused by the spread of *Perkinsus*. Other factors may also be responsible for the mortalities. Stress on abalone may result from environmental factors (e.g., pollutants, water temperature) with *Perkinsus* infecting abalone opportunistically. The pattern of spread of the mortality is most consistent with the spread of an infectious disease, such as that caused by *Perkinsus*, rather than other potential causes. Research done in South Australia, where *P. olseni* infections in blacklip and greenlip abalone (*Haliotis laevigata*) and associated mortalities have been documented, suggests *Perkinsus* may infect a wider range of species than previously thought. There are significant differences in the epizootiology of the disease observed in abalone in SA and NSW. In SA, in recent years, symptoms of infection by *Perkinsus* occur in abalone at a few localised sites and it does not appear that infections have spread beyond these locations. In contrast, the spatial and temporal pattern of reported mortalities between Port Stephens and Jervis Bay in NSW during the 1990's suggests that *Perkinsus* may have spread at this same scale. This is more similar to an outbreak of *P. olseni* that occurred in SA during the 1980s. The spread of reported mortalities in NSW may be due to a different species of *Perkinsus*, a more pathogenic strain of *P. olseni*, or related to interacting environmental factors. Mortalities may result from the spread of *Perkinsus* into naïve populations of abalone exposed to environmental conditions that make them susceptible. The threat of spread of *Perkinsus* to naive populations appears to be present in most abalone producing states of Australia.

Evidence from the USA, Europe and Asia, where *Perkinsus* spp. infect and cause mortality of other mollusc species, demonstrates that environmental variables (water temperature and salinity in particular) play an important role in the progression and regression of infection intensity. Stress in hosts, induced by unfavourable environmental conditions, limits the immune response of hosts. Abalone are easily stressed when exposed to a range of factors including high water temperature and poor water quality. It is likely, therefore, that such environmental factors may play an important role in the epizootiology of perkinsosis and observed mortalities of abalone in NSW.

Commercial and recreational fishers in NSW have been alarmed about the extent of the mortality observed since the early 1990's and the apparent failure of affected stocks to recover. In response to depleted populations of abalone, sections of the NSW coast were closed to fishing in 1996, 1998 and 2002, at which point, the entire section of coast between Port Stephens to Jervis Bay was closed to harvesting of abalone. Whilst a large proportion of abalone have died in some areas (greater than 95% at some sites since the mid 1990's), dense populations still occur in some limited areas within this range. Proposals for reopening of the area are currently being considered, but are being hampered by uncertainty about the extent of stocks that remain and the potential for transmission of *Perkinsus* to other areas.

NSW Fisheries (now within I&I NSW) convened a national workshop in September 2003 to help develop strategies for the research and management of the *Perkinsus*-related mortality of abalone. The workshop involved experts from state and commonwealth departments, Universities, FRDC and Industry, and concluded there was an urgent need to gain a greater understanding of the problem by advancing research in several directions. These research needs included the development of improved diagnostic assays for *Perkinsus* and investigations of resistance to the disease. The most urgent need identified, however, was the collection of data to further understanding of the cause of abalone mortalities observed in NSW and the pathogenesis and epizootiology of perkinsosis.

Despite the identification of *Perkinsus* in moribund abalone, it was not clear whether *Perkinsus* was responsible for the observed mortalities of abalone between Port Stephens and Jervis Bay or whether other environmental factors were involved. It was assumed, but not known, that the species of Perkinsus was *P. olseni*. Reports of the specific times and locations of mortality events were poorly documented. There was a strong and urgent need for basic information about historical mass mortalities. Identification of the current geographic range of *Perkinsus* infections in abalone was needed. Pathogenesis and epizootiology associated with *Perkinsus* infections and mortalities of abalone in NSW required formal investigation.

Outcomes of these investigations would directly aid current and ongoing management of the abalone fishery in NSW by providing information about: the cause(s) of the observed mass mortalities and specifically, the involvement of *Perkinsus*. Knowledge of the pathogenesis and epidemiology of perkinsosis would directly inform the need for further research and monitoring and management options for limiting further spread of *Perkinsus*. Such outcomes are likely to have implications for the research and management of all abalone fisheries in Australia that have been or might, in the future, be affected by *Perkinsus*-related mortality.

2. OBJECTIVES

(1) Compile and document the historical evidence about the spread of *Perkinsus*-related mortality of abalone in NSW.

(2) Describe the pathogenesis and make initial investigations of the epizootiology of the mortality of abalone with particular reference to the role of *Perkinsus*.

(3) Contribute to the development of strategies to manage populations of abalone that have, or might be, affected by *Perkinsus*-related mortality and, in particular, evaluate the need for a second phase of research.

These objectives were translated into more specific objectives that are grouped in chapters of this report as outlined below. It was also a general objective to provide a literature review pertinent to each topic within the introduction of each chapter.

Chapter 3 Reported mortality events and declines in abundance of abalone in NSW

(i) Investigate and document the historical observations of morbidity/mortality events by divers (by survey/interview of divers) and from records (Veterinary Laboratory reports and related documents) held by NSW DPI (now I&I NSW);

(ii) Collate and interpret data-series that provide indices of abundance of abalone over time (fishery-dependent catch and effort data, fishery-independent surveys of abundance, estimates of historical changes in abundance by divers) and model-based estimates of historical biomass.

(iii) Assess the relative importance of fishing mortality and other observed "mortality events" to historical declines in abundance of abalone.

Chapter 4 Identification and geographic distribution of the parasite *Perkinsus* sp. in abalone in NSW

(i) Survey the geographic distribution of *Perkinsus* sp. in abalone (in 2005) and extend the geographic range covered by the 2002-03 surveys;

(ii) Determine the spatial variability of prevalence and intensity of *Perkinsus* sp. infections in blacklip abalone (at spatial scales of: locations on the coast; sites within locations; areas adjacent to sites) and compare with results from 2002-03 surveys;

(iii) Assess the correspondence of positive and negative test results among 3 alternative diagnostic tests for the presence of *Perkinsus*: Ray's test, histology and PCR;

(iv) Positively identify the species of *Perkinsus* infecting abalone in NSW;

Chapter 5 Pathogenesis of *Perkinsus* sp. in abalone in NSW

(i) Investigate the distribution of *Perkinsus* sp. infections in organs/tissues of blacklip abalone;

(ii) Investigate the pathology and pathogenicity of *Perkinsus* sp. (tissue necrosis, changes in organ morphology and likely functional impairment of organs, host-response to infection);

(iii) Identify pathogens other than *Perkinsus* sp. (and describe associated pathology and pathogenicity if detected);

(iv) Assess the effect of *Perkinsus* sp. infection on the condition (weight-length relationship) of abalone;

(v) Assess evidence regarding the site (organ/ tissue) of initial infection and disease progression (based on the distribution of *Perkinsus* sp. in organs/ tissues from (i)).

Chapter 6 The epizootiology of perkinsosis and mass mortality of blacklip abalone in NSW

(i) Examine the effect of abalone gender on the prevalence and intensity of *Perkinsus* sp. infections;

(ii) Examine the effect of abalone size on the prevalence and intensity of *Perkinsus* sp. infections;

(iii) Implement a survey to monitor abalone abundance and mortality adjacent to the southern "front" of infection at South Jervis Bay;

(iv) Test for the presence of *Perkinsus* sp. in other potential mollusc hosts at locations where the parasite was present in abalone.

(v) Examine the potential for translocation of *Perkinsus* sp. during fishing and related activities;

(vi) Examine the potential for translocation of *Perkinsus* sp. during reseeding and enhancement experiments;

(vii) Identify environmental factors that were noted by divers to be associated with mortality events and changes in abundance of abalone;

(viii) Identify associations between water temperature, observed mortality events and infection intensity;

(ix) Investigate the availability of data about water quality and therefore, the potential to examine associations between water quality and observed mortality events.

(x) Consider the relative likelihood of alternative hypotheses about the epizootiology of perkinsosis and *Perkinsus*-related mortalities of blacklip abalone in NSW.

Chapter 7 Further development – management and research options

(i) Identify immediate management risks and options;

(ii) Identify high priority research and monitoring options that will inform management in the longer term.

3. REPORTED MORTALITY EVENTS AND DECLINES IN ABUNDANCE OF ABALONE IN NSW

3.1. Introduction

Changes in abundance of any species over time result from the net effect of factors that positively and negatively influence abundance. Growth of individuals and recruitment to the population has a positive impact on abundance and biomass. Removals of individuals from the population through fishing (fishing mortality) and non-fishing-related causes of mortality (natural mortality) decrease abundance and stock biomass. Fishing mortality may vary dramatically over time, initially as the fishery expands and catches increase and subsequently, if catches decrease as a consequence of over-fishing. Similarly, background levels of natural mortality may change as a consequence of changes in predator-prey relationships, environmental conditions (affecting reproduction, growth or survival) or the introduction of disease into the population. In attempting to examine the relative importance of changes in fishing and natural mortalities over time, establishing a timeline or history of fishing mortalities and other mortality events is fundamental.

The presence of dead or dying abalone on coastal reefs between Port Stephens and Kiama on the NSW coast between 1990 and 2000 has been reported by abalone divers on several occasions. These divers have also reported massive declines in abundance of abalone between Port Stephens and Kiama during the 1990s and 1999 – 2002 at Jervis Bay. Associated with several of these observations during the 1990's, moribund abalone were examined by the "NSW Fisheries Regional Veterinary Laboratory" at Wollongbar and *Perkinsus* sp. parasites were identified in some of these samples. Discussion concerning these observed "mass mortalities", subsequent depletions of abalone stocks and the role of *Perkinsus* has occurred in a variety of forums: meetings of the Abalone Management Advisory Committee (ABMAC); meetings of the Total Allowable Catch (TAC) committee; a workshop sponsored by FRDC and held at the Cronulla Fisheries Centre in September 2003; letters from fishers to NSW Fisheries and NSW DPI (now within I&I NSW); and discussions amongst scientists and managers within I&I NSW. Much of this discussion was based on 2nd- or 3rd-hand anecdotal information and undocumented interpretations of events. Thus, there was a clear need to: (i) formalise and document the recollections and observations of divers regarding morbidity/mortality events and declines in abundance at specific locations and at specific times; (ii) collate the information contained in reports from the NSW Fisheries Veterinary Laboratory (held by I&I NSW).

Understanding of the importance of such non-fishing mortalities, relative to the importance of fishing mortality, also requires assessment of the changes in abundance of abalone over the history of the fishery that are due to fishing. Stock assessments for the NSW abalone resource have been completed annually since 1995. These assessments have been based on: (i) fishery-dependent catch and effort data provided by commercial abalone fishers on logbooks; (ii) sampling size-distributions from commercial catches; (iii) a fishery-independent survey of the abundance of abalone on rocky reefs in NSW; and (iv) a model of the population and fishery that estimates historical changes in stock biomass (e.g., Worthington *et al.*, 1998, 1999, 2001; Worthington, 2003; Liggins and Upston 2006; Ferrell *et al.*, 2007). This model incorporates information about the biology of abalone (e.g., growth, size at maturity) and the fishery (e.g., selectivity, historical changes in minimum legal lengths) and is calibrated using time-series of fishery-dependent catch and effort data and standardised estimates of abalone abundance from the fishery-independent survey. Thus, depletions of abalone stock may be inferred from fishery-dependent catch and effort data, the fishery-independent survey of abundance or estimated biomasses from the model.

Management of the fishery for abalone in NSW is structured spatially into 6 regions, named Regions 1 – 6 from the north to the south of NSW (Fig. 3.1). Each region is further subdivided into zones and sub-zones. The component of Region 1 between Port Macquarie and Jervis Bay is of particular interest in this project and comprises Zones D, E, F, G, H, J, K and L from north to south (Fig. 3.1). Prior to 1995, fishers reported catch at the level of zone. Reporting at the level of sub-zone was phased in during 1995 and by 1996 all fishers reported catch and effort at the level of sub-zone (36 sub-zones within Region 1).

The fishery for abalone in NSW is a share-management fishery in which a total allowable commercial catch (TACC) is set annually by a TAC committee (NSW Fisheries, 2000; TEL, 2007). The fishery became the first restricted fishery in NSW in 1980. In 1989, a quota system was introduced with an annual quota of 10 t allocated to each diver. This led to average total catches of approximately 350 t (Andrew *et al.*, 1996). Following industry restructure, the TACC set for the fishery was 333 t between 1992 and 1999 but, in response to increasing concerns about abundance of abalone and sustainability of the fishery, decreased to 305 t for 2000, 300t for 2002, 281 t for 2003-04, 206 t for 2004-05, 130 t for 2005-06 and 125 t for 2006-07. A minimum legal length (MLL) of 100 mm was first introduced in 1973 and was increased to 108 mm in 1980, 111 mm in 1986 and 115 mm in 1987. Due to concerns about depletions of stock at specific locations within Region 1 during the 1990s, fishing closures were implemented for Sub-zones G3, H3, J1 and J2 in August 1996 and in Sub-zone K3 in December 1998. Subsequently, following further concerns about depleted stocks in the southern component of Region 1 between Port Stephens and Jervis Bay (Zones F – L), this component of the fishery was closed to fishing in November, 2002 and has remained closed since. A limited catch of 3t was taken from Zones G – L by commercial divers with research permits during 2004 and a further 3t from Zones F – L during 2007.

One of the consequences of the total closure has been that our knowledge of recent changes in abundance of abalone is inferior in Region 1 compared to Regions 2 – 6. Fishery-dependent catch and effort data is absent for the southern component of Region 1 since 2003. Moreover, whilst the fishery-independent survey of abundance, that commenced in 1994, was maintained in Region 1 up until 2007, the coverage of this relatively long distance of coastline by the limited number of survey sites was not as great compared to Regions 2 – 6. Indeed, no model-based estimates of biomass depletion have been made for Region 1 since 2000 (Worthington *et al.*, 2001). Biomass of legal-length ($\geq$ 115 mm) abalone had declined by an estimated 82% between the 1960's and 2000 (B_{2000} /B_{virgin} = 0.18, 90% C.I: 0.09 – 0.35). Subsequent assessments have discussed the declining status of stocks in Region 1 based on the catch and effort data and indices of abundance from the fishery-independent survey (e.g., Worthington, 2003) and data from the fishery-independent survey alone in years since 2003 (e.g., Liggins and Upston 2006; Ferrell *et al.*, 2007).

There is little doubt that there has been a massive decline in the abundance of abalone stocks along the NSW coast during the past 20 – 30 years, particularly so in the component of the fishery between Port Stephens and Jervis Bay (e.g., Andrew *et al.*, 1997; Worthington, 2003; Liggins and Upston 2006; Ferrell *et al.*, 2007). The decline in this latter component of the fishery could be due to a range of impacts including fishing, environmental factors (e.g., habitat changes, pollutants, water temperature) and disease (e.g., perkinsosis).

The principal objectives of this component of the project, presented in this chapter, were to:

(i) Investigate and document the historical observations of morbidity/mortality events by divers (by survey/interview of divers) and from records held by I&I NSW (Veterinary Laboratory reports and related documents).

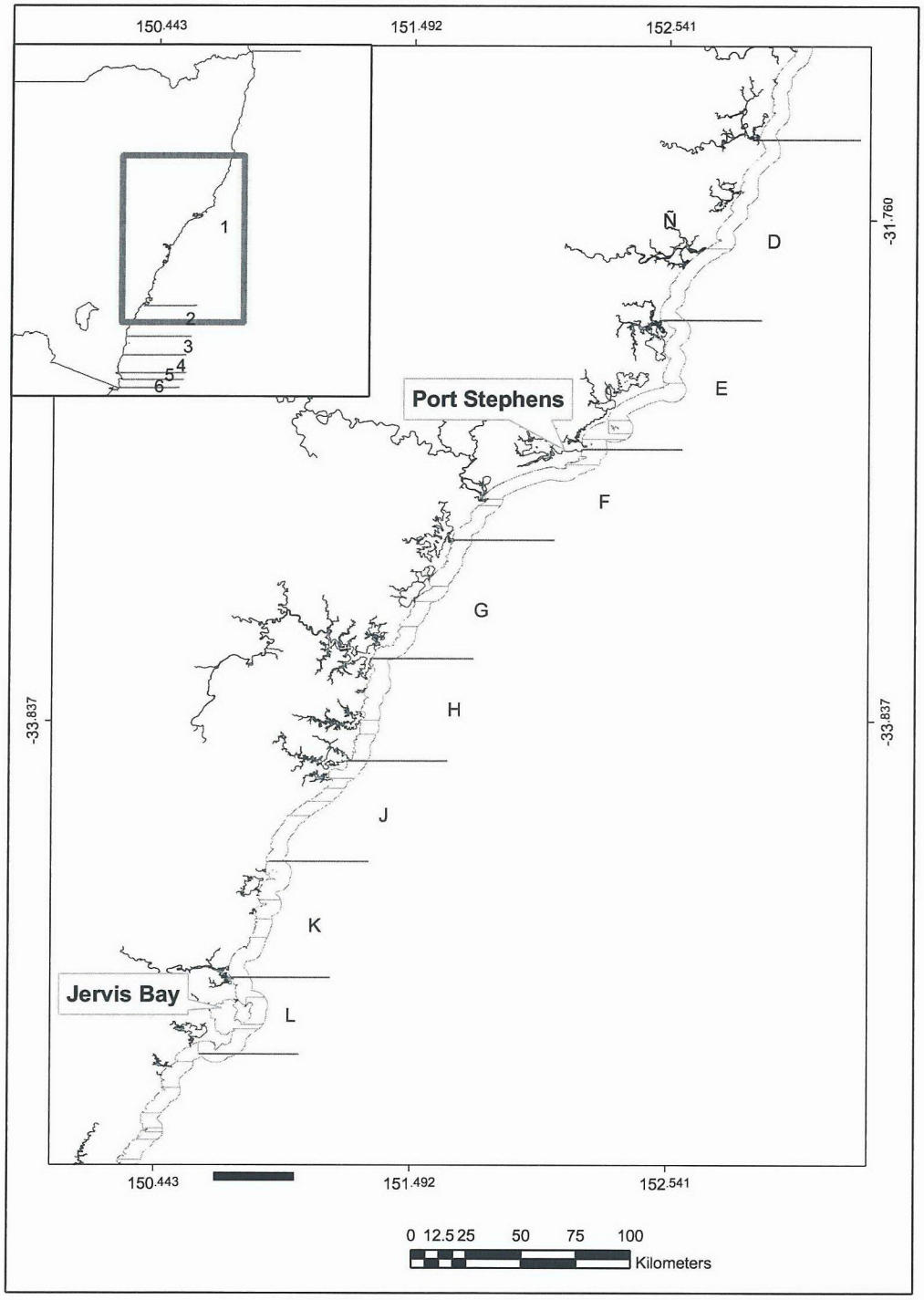

Figure 3.1. Regions 1 – 6 (inset) and main fishing zones within Region 1 (Zones D – L) of the abalone fishery. Horizontal lines within zones denote boundaries of sub-zones.

(ii) Collate and interpret data-series that provide indices of abundance of abalone over time (fishery-dependent catch and effort data, fishery-independent surveys of abundance, estimates of historical changes in abundance by divers) and model-based estimates of historical biomass.

(iii) Assess the relative importance of fishing mortality and other observed "mortality events" to historical declines in abundance of abalone.

3.2. Methods

Observations of historical morbidity/mortality events and sudden declines in abundance of abalone were obtained by interviewing divers with a history of harvesting abalone in Region 1. Diver observations of mortality/ morbidity events were verified against records held by I&I NSW (laboratory reports from the NSW Regional Veterinary Laboratory, Wollongbar, and associated documentation) where possible.

Three sources of potentially useful indices of abalone abundance were investigated: (i) estimated historical abundances from diver interviews; (ii) commercial catch and effort data (provided by commercial abalone fishers in NSW and held by I&I NSW); and (iii) fishery-independent surveys of abundance completed by NSW Fisheries / NSW DPI (now I&I NSW) and done under contract by The Ecology Lab Pty. Ltd. during 2005 and 2006). Model-based estimates of historical biomasses and depletions were obtained from resource assessments (completed by NSW DPI, now I&I NSW).

3.2.1. Diver interviews (observations of morbidity/mortality events, sudden declines in abundance, historical changes in abundance)

A total of 17 commercial and 9 recreational abalone divers with a history of harvesting abalone in Region 1 (Port Stephens to Jervis Bay) during the period 1975 to 2003 were selected for full interviews, which were done face-to-face. An additional 6 part-interviews (5 commercial and 1 recreational fisher), which focussed on a sub-set of questions relating to observed mortalities, were done over the phone. The criteria for selection of commercial divers for full interviews were either a consistent diving history within Region 1 or large catch weights over fewer years. Divers were either resident within Region 1 where most of their catch was taken, or were "travelling" divers who made regular trips from southern ports to fish certain areas within Region 1. The coverage of full and part interviews within Region 1, based on the divers' main area fished, was as follows:

- Port Stephens (Zone F): Total of 14 interviews, 7 commercial and 7 recreational divers;
- Central Coast (Zone F, G): Total of 14 interviews, 11 commercial and 3 recreational divers;
- Sydney to Wollongong (Zone H, J): Total of 14 interviews, 11 commercial and 3 recreational divers;
- Kiama (Zone K): Total of 15 interviews, 13 commercial and 2 recreational divers;
- Jervis Bay (Zone L): Total of 15 interviews, 13 commercial and 2 recreational divers.

Divers were independently asked a series of questions relating to abundance of abalone and observed mortalities within Region 1, and factors which may have impacted on the abundances such as environmental changes (Appendix 3 in Section 12.3). The same person interviewed each diver in one or two sessions for 4 – 6 hrs if a commercial diver, or approximately 2 hrs if a recreational diver. Two different sets of interview questions were constructed for commercial and recreational divers; however there was still considerable overlap in the questions asked. Relevant

to this study were questions about (i) relative abundance of abalone over time, (ii) sudden declines in abalone abundance (i.e., within one year), and (iii) observed dead or dying abalone. Specifically, the questions were as follows:

(a) Commercial and recreational divers (Questions 12 (a) and 6 respectively)

How would you describe change in abundance of abalone in Region 1, for each year fished, up until the present? (Answer is a graphic of % abundance over time)

- Full interviews only;
- For commercial divers abundance related to legal-size at the time of fishing and for recreational divers abundance referred to abalone reliably seen within cryptic reef habitat (> 80 mm shell length for most of the divers interviewed);
- If the change in abalone abundance was judged by a diver to be different for their main areas fished then separate graphics were drawn for each area;
- Not all divers could confidently recall abundance of abalone through time for each of their main areas fished. Typically "travelling" divers recalled abundances for a sub-set of their main areas fished. The coverage of graphics based on the divers' ability to recall abundances for their main area fished, was as follows: Port Stephens, 8 (3 commercial, 5 recreational); Central Coast, 7 (6 commercial, 1 recreational); Sydney to Wollongong, 4 (2 commercial, 2 recreational); Kiama, 8 (6 commercial, 2 recreational); and Jervis Bay, 4 (commercial);
- Indices of abalone abundance for each fishing area were calculated from the average change in each year relative to the previous year.

(b) Commercial divers (Questions 10 and 12 (d))

Specify exceptional years when abundance of legal size abalone in Region 1 was either very good or poor?
AND
Were there specific areas you fished, where abalone abundance declined suddenly (within the space of months)?

(b) Recreational divers (Question 7)

Were there specific years when relative abundance of abalone had either noticeably increased or decreased?

(c) Commercial and recreational divers (Questions 16 and 10 respectively)

Did you observe abalone dead, or with abnormal signs (e.g., lesions, lethargic), on reefs in Region 1? If so please provide details and specify year, area and approximate number of individuals observed.

For questions under categories (b) and (c), the certainty of diver recollections for years in which mortalities or sudden declines in abundance were noticed was documented as either approximate with a minimum error of +/- 1 year (e.g., ~2000), or certain if mortalities occurred within a specified year or range of years for which the diver was certain (e.g., 1999 or 2000).

Diver observations of morbidity/mortality events or sudden declines in abundance were summarised and tabulated. The observations or mortalities were verified by searching records held by I&I NSW for reports of abalone mortalities (Fishkill database, all records since early 1970's) and examinations of abalone done by the Regional veterinary Laboratory at Wollongbar (records

for submissions since 1986 and reports including the text "abalone" since 1990). Laboratory reports that corresponded to diver reports of mortalities (i.e., year and area matched) provided additional information such as whether *Perkinsus* or other pathogens were detected in abalone at the time.

3.2.2. *Fishery-dependent catch, effort and CPUE*

Divers are required to provide information on their daily catch and effort within each of the sub-zones of the fishery. Prior to 1996 catch was reported at the level of zone only. Catch (kg) is validated each day and effort is estimated as diving hours. Catch, effort (hours) and CPUE (catch per hour) since 1983 are described for Region 1 of the fishery, northern (Zones A – E) and southern (Zones F – L) components of the region and for each of the individual Zones F – L (between Port Stephens and Jervis Bay). Annual CPUE for each region was calculated as the sum of catch (kg) / sum of effort (hours fished). Note that a total closure of the fishery between Port Stephens and Jervis Bay has been effective since November 2002 so that this data series covers the period 1983 – 2002.

3.2.3. *Fishery-independent surveys of abundance*

Fishery-independent surveys of the abundance of abalone on reefs within NSW were done annually between 1994 and 2007 to estimate the abundance of small (< 60 mm), medium (≥ 60 mm and < 115 mm) and large (≥ 115 mm) abalone (e.g., Worthington *et al.*, 1998; Worthington, 2003; Liggins and Upston, 2006). Within Region 1, counts of abalone have been made annually since 1994 at up 74 fixed areas ("crevices") between Port Stephens and Jervis Bay. These fixed areas are grouped within sites, typically with 2 – 6 fixed areas per site. Sites are grouped within each of four locations, Port Stephens (Zone F), Sydney (Zone J), Kiama (Zone K), and Jervis Bay (Zone L). For each fixed area, two replicate counts, usually by different divers but rarely by the same diver, were made of abalone in each of the three length classes. The sum of these counts then provided a total count for each fixed area. Note that the number of fixed areas sampled varied from year to year but in later years was greater than when the survey began in 1994 as additional crevices have been added over time. Also note that Jervis Bay was first sampled in 2002.

To calculate indices of the relative abundance of abalone in the fixed areas within zones of Region 1, a generalised linear model was developed (Worthington and Andrew, 1997; Worthington *et al.*, 1998). This model describes the number of abalone in each area as a series of independent Poisson variables, with a $\log_e$ link, and an additional parameter to account for over-dispersion of the observations. Variables included in the model are year, zone, area and diver for each count to enable a parameterisation which summarises the number of abalone in each year and region as a proportion of the initial number of abalone in each area. Time-series of indices of abundance from these surveys have been presented annually in resource assessments (e.g., most recently in: Liggins and Upston, 2006; Ferrell *et al.*, 2007).

These data are presented for each of the 3 regions (Port Stephens, Sydney and Kiama) for which time series exist for the period 1994 – 2005.

3.2.4. *Comparisons among alternative indices of abundance and model-based estimates of biomass*

Correspondence between annual commercial catch since 1983; abundance of abalone (all size-classes) relative to 1994 from independent surveys; abundance of abalone (≥ 100 mm length) relative to 1975 from diver interviews was examined graphically and aided by the calculation of correlation coefficients. The range of years over which each comparison could be made was limited by the overlap of the relevant data series: commercial catch v independent surveys (1994 – 2002); commercial catch v diver interviews (1983 – 2002); independent surveys v diver interviews

(1994 – 2003). Note that for Zones G and L, where little or no information exists from independent surveys, only the comparison between commercial catch v diver interviews (1983 – 2002) was done.

3.3. Results

3.3.1. Diver interviews – morbidity/mortality events and sudden declines in abundance

Observations relating to morbidity and mortality of blacklip abalone and sudden declines in abundance are summarised in Table 3.1. An important aspect of this summary table is degree of certainty associated with recollections of specific years by divers. Many divers reported that observed events occurred in "approximate" years or provided a range of years. The years associated with documented reports from the NSW Fisheries Regional Veterinary Laboratory are particularly reliable and are highlighted. Also highlighted are the ranges of years in which morbidities/mortalities of "100s" or "1000's" of abalone were reported by divers, but restricted to events that were not associated (by the divers) with cyclones, floods or mortalities of non-mollusc species such as urchins.

Port Stephens

Only 4 out of 14 divers who specified Port Stephens as one of their main diving areas reported dead or dying abalone. Each of these 4 divers observed dead or dying abalone in F1 and the northern part of F2 (Yacaba Head to One Mile Beach). One of these divers reported deaths (but the scale of the event was not specified) across all size classes at Boulder Bay in approximately 1994 and a single "sick" abalone on the south side of Fingal Island in approximately 1994.

The only observations of mortalities on a large scale (i.e., specified by divers as 100's – 1000's of abalone) occurred around 2000. Two divers reported mortalities on this scale in Sub-zones F1 and F2 (southern portion of Port Stephens), one nominating the year as 1999 or 2000 and the other approximately 2000. A third diver observed "sick" abalone in the same area in approximately 2000. Sudden declines in abundance of abalone at the scale of 100's – 1000's were reported for the years 2000, 2001 and possibly 2002. For subsequent use in this report, these observations are collectively referred to as the **"2000 mortality event" at Port Stephens**.

There were no samples of morbid or dead abalone taken from Port Stephens for further investigation.

Central Coast

More than half the number of divers (8 out of 15 who specified the Central Coast as a main fishing area) observed dead or dying abalone in this area, mostly in Sub-zones G3 and G4 (Terrigal to Broken Bay). Mortalities in the early to mid 1980's were reported by 3 divers. One diver reported 100's of abalone dead after cyclones in the early 1980's. Another diver reported 1000's of dead abalone and sea-urchins in approximately 1983 – 85 in the Terrigal to Broken Bay area (Sub-zones G3 and G4). A third diver reported a small-scale mortality (< 10 abalone) of abalone washed up on Shelly beach (Sub-zone F4).

Table 3.1. Mortality / morbidity events and sudden declines in abundance of abalone reported by divers during interviews and based on laboratory reports from the NSW Fisheries Regional Veterinary Laboratory, Wollongbar. * denotes a non-commercial diver; ~ denotes approximate year; dark-grey shading denotes information from a laboratory report; light-grey shading denotes the range of years in which mortality / morbidity events of order 100's or 1000's were reported (that were not associated with cyclones, floods or mortalities of urchins).

Location		Diver	Mortality / Morbidity					Sudden decline	Comments
Subzone	Area		Year	Season	Scale	Size-class	Other Spp	Year	
Port Stephens									
F1, F2	PS sth	M	-	-	-	-	-	1991	
F1	Boulder Bay	T	~1994	-	-	all	-	-	
F1	Sth Fingal Is	T	~1994	-	<10	all	-	~1992-1996	Localised decline ~1992 to 1996 (illegal fishers)
F1, F2	PS sth	MM*	-	-	-	-	-	1990s	
F1, F2	PS Sth	GF*	-	-	-	-	-	1997 or 1998	
F1, F2	PS sth	BD	1999 or 2000	winter	100's to 1,000's	all	-	1999 or 2000	Some of dead abalone with research tags from reseeding progran
F1, F2	PS sth	T	-	-	-	-	-	2000	Decline in large-size abalones (100's to 1000's)
F1, F2	PS sth	GF*	~2000	winter	100's to 1,000's		-	~2000	Large seas ~2000
F1, F2	PS sth	MM*	~2000 ("sick" only)		10's		-	-	Abalones "shrinking" in their shells
F1, F2	PS sth	BB*	-	-	-	-	-	2001	
F1, F2	PS sth	SB*	-	-	-	-	-	~2001	Large decline (1000's)
F1, F2	PS sth	JS*	-	-	-	-	-	Dec 2001 or 2002	Large decline (1000's)
Central Coast									
G	Swansea to Broken Bay	A	early 1980's	-	100's	all	-	-	Cyclone
G3,G4	Terrigal to Broken Bay	AI	~1983 or 1985	-	1000's	all	Urchins, Turbans	mid 1980's	Dead & dying molluscs at STP outlet (poor water quality?)
F4	Shelly Beach	BH*	mid 1980s	-	<10	large	-	1980's	
G3	Terrigal (Skillion)	A	-	-	-	-	-	1985	
G1, F3, F4	Swansea/ Newcastle	T	-	-	-	-	-	1986	
G3	Terrigal	LG*	-	-	-	-	-	by ~1988	
G3	Terrigal	BH*	-	-	-	-		~1990	Decline ~ 1990 followed sand-up event
G3	Terrigal	BM	1991_or_1992	summer	1000's	>75 mm	-	1991 or 1992	
G3	Terrigal (sth of STP) to Sydney	AL	1992	-	10's to 100's	-	-	-	Mortalities first noticed at Terrigal
G3	Terrigal (The Skillion)		1992	May					(LAB. REPORT, 1992) Perkinsus sp. in 2/2 abalone Assoc. with mortality event
G3	Terrigal	BO	1992 or 1993	summer	1000's	most large	-	-	
G3	Terrigal (Skillion sth)	BN	1992 or 1993	-	1000's	all	-	1992 or 1993	
G4	Little Beach (nth of Hawkesbury)	AX	~1994 or earlier	-		>50 mm	-	-	
G1	Swansea	BN	-	-	-	-	-	1994 or 1995	
Sydney - Wollongong									
J2-J5	Port Hacking to Wollongong	K	-	-	-	-	-	1987	Decline in abalones Port Hacking to Wollongong
H1	North Head	AX	~between 1987-1991	-	-	-	-	-	
H1	North of Sydney	AL	-	-	-	-	-	-	~1992-1994 10's to 100's empty shells nth of Sydney
H1,H2	Sydney Heads	AB*	1993	summer			-	by 1993	Localised declines at some spots (100's)
J3-J5	Coalcliff (& Era - Coledale)		1993	May					(LAB. REPORT, 1993) Perkinsus-like organism in 1/4 ab. from Coalcliff; assoc. mort. event Era-Coledale
J5	Coledale	AN	~1994 or 1995	summer	-	all	-	-	
		BV*	-	-	-	-	-	-	~1996 empty shells Little Marley to Stanwell (illegal fishers?)

Table 3.1. (Cont'd)

Location		Diver	Mortality / Morbidity					Sudden decline	Comments
Subzone	Area		Year	Season	Scale	Size-class	Other Spp	Year	
Kiama									
K2	Minamurra (off land mass)	K	**1972**	-	1000's	all	-	-	Floods
K4	Gerringong	E	-	-	-	-	-	1986-1988	Abalones slow growing, 6 mo instead of 6 wks to reach legal-size
K3,K4	Kiama	BV*	-	-	-	-	-	~1988	
K2	Minamurra	O	bet. 1988-1989 (abnormal only)	-	-	-	-	-	Abalones with unusual marks on foot & deformed shells
K3,K4	Kiama	BV*	-	-	-	-		~1992	
K2	Minamurra	E	-	-	-	-	-	by ~ 1992 or 1993	
K4	Walkers Beach	M	**~1994**		100's to 1000's		-	~1994	Large decline over 3 months
K3	Railway Bay	BO	**1995 or 1996**				-		
K3, K4	Kiama		**1996**	April					**(LAB. REPORT, 1996) Perkinsus sp. detected in 10/13 abalone; Live samples following mortality event**
K3	Loves Bay	X	-				-	1995 or 1996	Large decline in juveniles (1000's & 1000's); empty shells seen
K3,K4	Kiama	BP	**1996 or 1997**		100's	large	-		Dozens of dead/dying abalones at several spots
K4	Gerringong to Crookhaven Bight	AI	-	-	-	-	-	1990's	empty shells seen (100's to 1000's), all sizes
K4	Gerrora/Black Head	K	~1998 (abnormal only)	-	-	-	-	-	Abalones shells deformed
K4	Walkers nth	X	-	-	-	-	-	~1998	No abalones at best drops
K3,K4	Kiama	BN	-	-	-	-	-	by 1998	
K3,K4	Kiama	BN	-	-	-	-	-	-	In 2000 empty shells seen (1000's), all sizes
Jervis Bay									
L4	Stoney Creek	BN	-	-	-	-	-	-	Bet ~2000 to 2001 empty shells seen (1000s)
L4	Sth Stoney Creek (Cape St George)	CL	-	-	-	-	-	-	In ~ 2001 empty shells seen (100s), all sizes (suspect poisoned?)
L4	Stoney Creek (centre of bay)	CL	-	-	-	-	-	-	In 2000 or 2001 empty shells seen, all sizes
L4	Nth Stoney Creek	BM	-	-	-	-	-	-	Empty shells seen, all sizes (suspect poisoned?)
L4	Nth Stoney Creek (old lighthouse)	CP	-	-	-	-	-	-	Empty shells seen (<10), all sizes
L2 & L4	Jervis Bay	BN	-	-	-	-	-	2001	Dramatic decline (concentrated commercial fishing)
L2 & L4	Jervis Bay	CL	-	-	-	-	-	2001	Large decline (100's to 1000's); abundance 50% of previous year
L4	Bowen Is to Wreck Bay	CP	-	-	-	-	-	2002	Decline (100s) noticed over 6 months

During the period 1991 – 1993, 4 divers observed large-scale mortalities of abalone (100's – 1000's) between Terrigal and Broken Bay (southern part of Sub-zone G3 and in G4). One diver was certain of his observation in 1992. The other 3 divers recalled that mortalities occurred sometime within a 2-year period (1991-92 or 1992-93). A fifth diver also nominated mortalities in this area in approximately 1994 or earlier. Two dead abalone were taken from the base of "The Skillion" at Terrigal and were examined at the NSW Agriculture Regional Veterinary Laboratory at Wollongbar. The laboratory report (Laboratory report WN92/1337, see Appendix 4 in Section 12.4.1) states that *"Other examples were seen to be lying on the bottom shell down either dead or near death. The bottom was littered with a large number of empty shells. Fish activity appeared to be normal. Ocean temperature was warm."* Following histological examination, *Perkinsus* sp. was identified in both specimens and the report concluded *"Suspected Perkinsus* sp. *infection. The contribution of this infection to the mortality problem is uncertain."*.

The mortality event or events between approximately 1991 and 1993 observed by divers on the Central coast and confirmed by the "history" statement in the laboratory report in 1992 is subsequently referred to in this report as the **"1992 mortality event" on the Central Coast**.

Sydney to Wollongong

Only 3 out of 14 divers who specified Sydney to Wollongong as one of their main diving areas reported observations of dead or dying abalone. None were specific about the scale of the mortalities observed. One diver reported recently dead abalone at North Head, some time between 1987 and 1991. A second diver observed dead/dying abalone during 1993 at Sydney Heads. Another diver reported dead abalone in approximately 1994-95 to the south of Wattamolla near Coledale (J5). Divers also noted seeing patches of empty shells (10's to 100's) at locations where mortalities had been reported (Sub-zone H1 in approximately 1992 – 1994; J3 and J4 ~1996).

In May 1993, following reports of dead and dying abalone between Era and Coledale (Sub-zones J3 – J5) a sample of 4 abalone specimens was collected offshore from Coalcliff (Sub-zone J5) from an area containing "dead shell". One of the 4 abalone examined by the Veterinary Laboratory contained *Perkinsus*-like organisms and the description of histology is consistent with *Perkinsus* trophozoites (Laboratory report WN93/1341, see Appendix 4 in Section 12.4.2).

The mortality event or events observed by divers around 1993 in this area are subsequently referred to in this report as the **"1993 mortality event" in the Sydney-Wollongong area**.

Kiama

Four out of 15 divers who specified Kiama as one of their main diving areas reported dead or dying abalone. One diver observed a large-scale mortality of abalone off Minnamurra (K2) in 1972 associated with major storms and flooding of the river.

The second and broad-ranging event occurred in Sub-zones K3 and K4 in the mid 1990's. Three divers observed dead or dying abalone between 1994 and 1997 (one nominated approximately 1994, one nominated 1995 or 1996 and another 1996 or 1997). Two divers nominated the scale of these mortalities in the 100's or 1000's. Two divers reported specific locations (Walkers beach in Sub-zone K4, Railway Bay in Sub-zone K3). Other locations where 1000's of empty abalone shells and co-incident localised declines were observed by 2 divers included Love's Bay (Sub-zone K3, summer 1995 or 1996) and Walkers (Sub-zone K4, approximately 1998 and in 2000).

Following a reported mortality event in 1996, a total of 13 abalone were collected from several locations within the Kiama area and examined by the Veterinary laboratory. *Perkinsus* infections were detected in 10 of the 13 abalone examined. The report (WN96/1001, see Appendix 4 in

Section 12.4.4) states that in some specimens there was "severe inflammation and necrosis" associated with the infections.

The mortalities observed by divers at Kiama in the mid 1990s are subsequently referred to in this report as the **"1996 mortality event" in the Kiama area.**

Jervis Bay

None of the 15 divers who specified Jervis Bay as one of their main diving areas reported dead or dying abalone. However, 6 divers noticed up to 100s or 1000's of empty abalone shells at one location on the southern side of Jervis Bay at Stoney Creek in 2000 or 2001 (Sub-zone L4). Curiously, 2 divers reported that they found 6 to 12 plastic bottles with holes drilled in the lids, wedged under ledges at this location. These divers raised the possibility of poisoning based on these observations.

Sudden large-scale declines in abundance of abalone were observed by several divers during 2001 and 2002. Because no direct observations of morbidity or mortality were made at Jervis Bay, the sudden declines in abundance observed at this location are subsequently referred to in this report as the "2000 – 2002 sudden decline in abundance" at Jervis Bay.

3.3.2. Fishery-dependent catch, effort and CPUE

Catches from the southern component of Region 1 (Zones F – L, i.e., Port Stephens to Jervis Bay) dominated the total catches of abalone from Region 1 during the period 1983 to 2002 (Fig. 3.2). During this period, annual catches from the southern component (Zones F to L) represented greater than 77% of the total annual catches from Region 1. It was only in 2003, following the closure of Zones F to L that the relatively small catches from the northern part of Region 1 (Zones A to E) became dominant – Zones A to E being the only component of Region 1 open to fishing.

Annual catches from the southern component (Zones F – L) of the fishery in Region 1 have decreased from 125 t in 1983 to 4 t in 2002 (Fig. 3.2). Note that the minimum legal length for abalone increased in 1986 and again in 1987 and that the introduction of quotas and total allowable commercial catches (TACCs) would have had a negative impact on catches during this period (Fig. 3.2).

The close relationship between catch and effort between 1983 and 2003 for Region 1 is particularly striking (Fig. 3.2). This is apparent for both the northern and southern components of Region 1. The positive correlation between annual catch and effort for the southern component of Region 1 (Zones F – L) between 1983 and 2002 was 0.95 ($P < 0.01$, $R^2 = 0.89$). CPUE (kg/hr) was relatively constant across the period 1988 to 2002, fluctuating within the range 18.3 – 25.4 kg/hr with a mean 22.0 kg/hr. Prior to 1988, when the current minimum legal length of 115 mm was introduced, CPUE was greater and up to a maximum 35.8 kg/hr in 1984 (Fig. 3.2).

Catch, effort and CPUE data for individual zones in the southern section of Region 1 (Zones F – L) are shown in Figure 3.3. Annual catches and effort were positively correlated for each of the 6 zones (R in range 0.93 – 0.96, R^2 in range 0.86 – 0.93, $P < 0.01$ for each zone). Consequently, as for the catch and effort data summed across these zones, CPUE within each zone was relatively constant during the period 1988 – 2002, having decreased from greater levels during the preceding 5 years (Fig. 3.3).

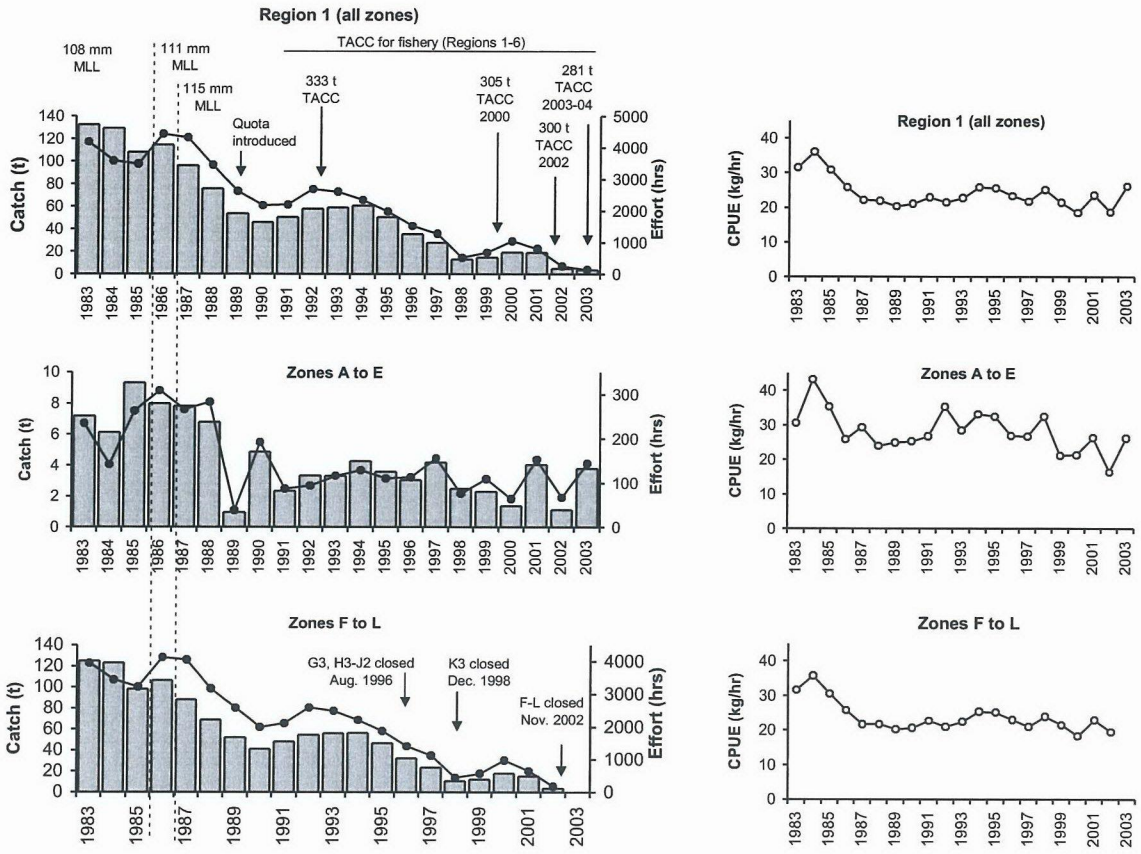

Figure 3.2. Total reported commercial catch of abalone (grey bars), effort (black filled circles) and commercial CPUE (kg/hr, circles) since 1983 for Region 1 (top panels), Zones A to E (middle panels) and Zones F to L (bottom panels). Major management changes (minimum legal lengths and TACCs) in the fishery are annotated. Note that an area-based TACC was set for Region 1 (19 t) and for Regions 2 – 6 (286 t) during the years 2000 and 2001.

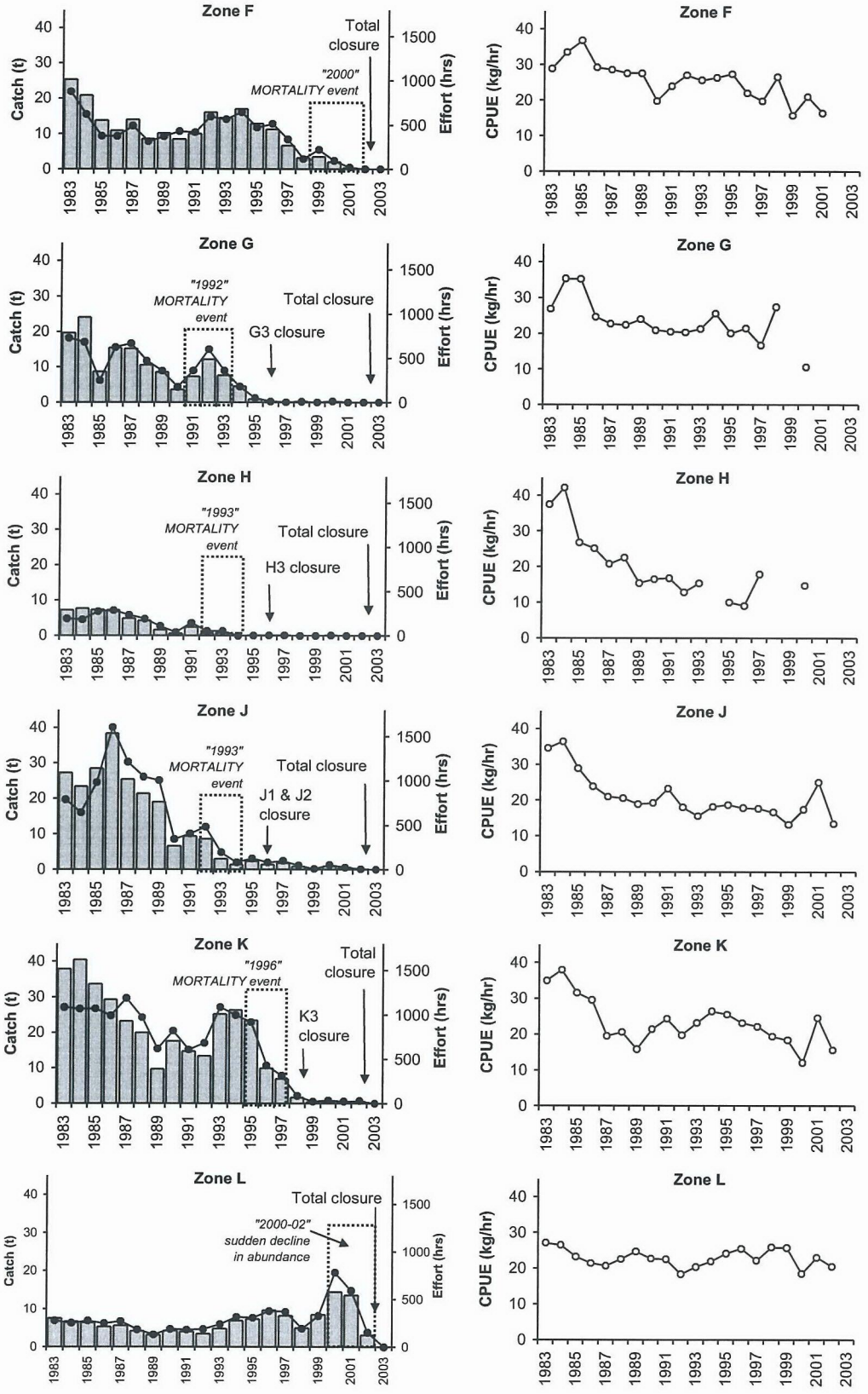

Figure 3.3. Total reported commercial catch of abalone (grey bars), effort (black filled circles) and commercial CPUE (kg/hr, open circles) since 1983 by zone.

There is, however, a difference among the patterns of catch (and effort) for Zones F – G. During the 1980's, significant catches came from all zones. By the mid 1990's, effort in Zones G, H and J had declined markedly, with the 1995 catch from these zones representing only 1 – 9% (Zone G: 9%; Zone H: 1%; Zone J: 9%) of the mean annual catch during the 1980's. In the mid 1990's catch and effort in Zones F and K dominated the region. By the late 1990's catch and effort had declined in Zone F and Zone K with the majority of effort being expended in Zone L, 90% of the catch coming from Zone L by 2001. In the first 10 months of 2002, prior to the total fishing closure implemented for Zones F – L in November, the total catch of 3.7 t from this component of Region 1 represented just 3.9% of the average catch achieved during the 1980's.

The pattern is one of progression of effort and catch from zone to zone. Declines in effort and catch in Zones G, H and J (Central Coast, Sydney, Wollongong) by the early 1990s were followed by increases in effort and subsequently catches from Zones F and K (Port Stephens and Kiama) in the mid 1990s. Declines in catches from these zones in the late 1990's was followed by an increase in effort into Zone L (Jervis Bay) during 1999 – 2001, effort and catch then declined here in 2002.

If annual catch within a zone is used as an index of abundance of abalone, then ratios of catch calculated between the commencement of the time-series of catch data (1983) and the year prior to the nominated mortality events identified for each zone, provide estimates of biomass depletion (Table 3.2). Note that depletion was not estimated for Zone L because catches increased in this zone during 2000 and 2001. Depletions of biomass since mortality events, based on annual catches, were not calculated because the closures to fishing implemented in 1996, 1998 and finally the closure of Zones F – L in 2002 resulted in zero catches independent of abundance.

Estimated depletions of abalone prior to the nominated mortality events in each zone ranged from 0.14 – 0.62 with a mean depletion of 0.35 (Table 3.2). Thus, an estimated 65% of the biomass of abalone in 1983 was removed prior to the mortality events identified in Zones F – K of Region 1.

Table 3.2. Depletions of catch prior to significant mortality events identified for zones within Region 1.

Zone	Year of "Mortality Event"	Depletion of catch PRIOR TO "mortality event"	Depletion of catch SINCE "mortality event"
Zone F Port Stephens	"2000"	C_{1999} / C_{1983} : 0.14	-
Zone G Central Coast	"1992"	C_{1991} / C_{1983} : 0.38	-
Zones H & J Sydney-W'gong	"1993"	C_{1992} / C_{1983} : 0.27	-
Zone K Kiama	"1996"	C_{1995} / C_{1983} : 0.62	-
Zone L Jervis Bay	2000 – 02 *		-
		Mean : 0.35	-

** sudden decline in abundance during 2000 – 02 for Zone L, Jervis Bay – there was no observed morbidity /mortality of abalone directly observed by divers and reported during interviews.*

3.3.3. Fishery-independent surveys of abundance

The distribution of fixed survey sites within Region 1 is shown in Figure 3.4. This clearly depicts the concentration of fixed sites adjacent to Port Stephens, southern Sydney, Kiama and Jervis Bay.

Abundance of abalone (all sizes combined) at sites in Zone F (Port Stephens), declined between 1994 and 2001 by approximately 89% (Fig. 3.5). Since 2001, depletions of abalone relative to 1994 have been remained between 7% and 24% of 1994 levels. Abundance of abalone at sites within Zone J (southern Sydney), declined between 1996 and 2006 by an estimated 65% (Fig. 3.5). The decline at survey sites in Zone K (Kiama) was approximately 73% between 1994 and 1999 and abundances since that time have remained between 28% and 33% of 1994 levels (Fig. 3.5).

At each of the 3 locations patterns of abundance of small and medium size-classes of abalone closely mirror the declines of all abalone (the 3 size-classes combined). This is because the counts of small and medium abalone are very much greater than counts of large abalone at the fixed sites (crevices). For example, in 1996, means of 52 small, 131 medium and 7 large abalone were counted at fixed sites in Region 1. Respectively, these counts represent 27%, 69% and 4% of the total counts of abalone. This explains why the great fluctuations in the abundance of large abalone (relative to 1994 at Port Stephens and Kiama, and relative to 1996 at Sydney) have negligible impact on patterns of abundance for total abalone (Fig. 3.5).

Based on indices of abundance of abalone from fishery-independent surveys at Port Stephens (Zone F) and Kiama (Zone K), depletions in the abundance of abalone may be calculated between the year prior to the nominated mortality events and survey year 2006 (Table 3.3). Depletions could not be estimated for Zones G and L due to the lack of survey sites in these zones. Depletions could not be estimated for Zones H and J because the fishery-independent survey did not commence until several years after the "1993 mortality event" at Sydney-Wollongong. Depletions of biomass prior to mortality events could not be calculated using data from independent surveys due to the absence of survey data prior to 1995.

Estimated depletions of abalone between commencement of mortality events and 2006 in Zones F (Port Stephens) and K (Kiama) were 0.29 and 0.23 respectively, with a mean depletion of 0.26 (Table 3.3). Thus, on average, an estimated 74% of the biomass of abalone present in the year prior to nominated mortality events, was removed (or died) by 2006.

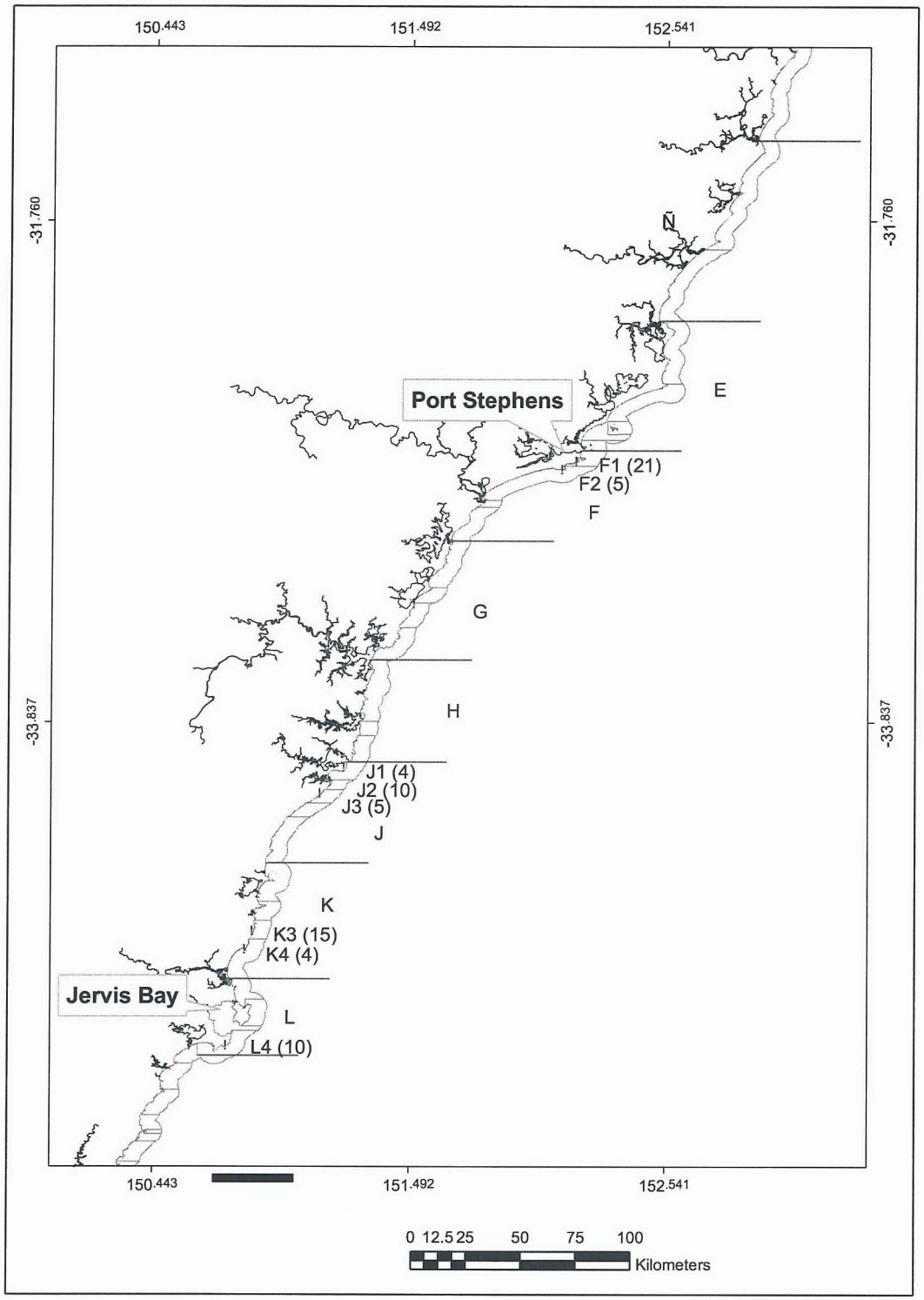

Figure 3.4. Map of NSW coast showing coverage of abalone independent survey sites within zones and sub-zones within Region 1. Number of fixed-sites per sub-zone is indicated in brackets.

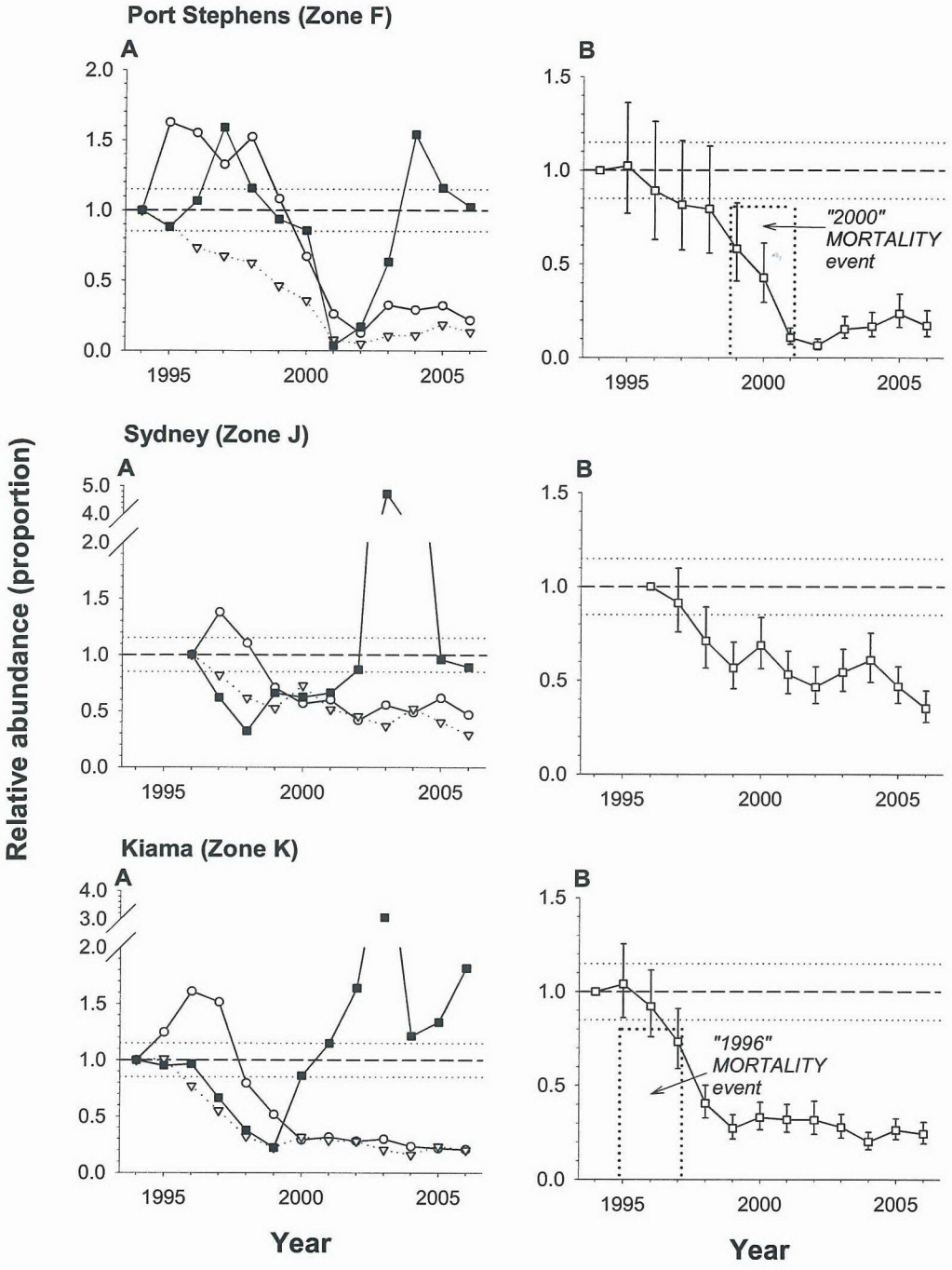

Figure 3.5. A. Abundance of small (circles), medium (triangles) and large (filled squares) abalone relative to 1994 or 1996 for each zone from the independent survey. B. Abundance of TOTAL abalone relative to 1994 or 1996 (+/- 95% CI). Dashed lines represent the value of the index in the first survey year, and dotted lines are +/-15%.

Table 3.3. Estimated depletions of abalone, based on indices of abundance from fishery-independent survey, since significant mortality events identified for zones within Region 1.

Zone	Year of "Mortality Event"	Depletion of Index PRIOR TO "mortality event"	Depletion of Index SINCE "mortality event"
Zone F Port Stephens	"2000"	-	I_{2006} / I_{1999} : 0.29
Zone G Central Coast	"1992"	-	-
Zones H & J Sydney-W'gong	"1993"	-	-
Zone K Kiama	"1996"	-	I_{2006} / I_{1995} : 0.23
Zone L Jervis Bay	2000 – 02 *	-	-
		-	Mean : 0.26

** sudden decline in abundance during 2000 – 02 for Zone L, Jervis Bay – there was no observed morbidity /mortality of abalone directly observed by divers and reported during interviews*

3.3.4. Historical trends in abundance from interviews of divers

Based on the structured interviews with divers who had histories of harvesting abalone in Region 1, indices of abundance relative to 1975 were calculated for Zones F – L (Fig. 3.6). Note that the numbers of divers contributing information to these indices in any given year in any given zone ranged between 1 and 7. Thus the reliability of the components of these time-series that are based on estimates from few divers must be regarded with caution. This particularly applies to the 1970's and 1980's for Zone F (Port Stephens) and Zone L (Jervis Bay).

The overall pattern is one of declining abundance of abalone within each zone between 1975 and the 2003. Importantly, relative to 1975, these indices of abundance based on the observations of divers interviewed suggest substantial depletions of stocks prior to the mortality events observed by these same divers (Fig. 3.6). The accuracy of the estimated depletion in Zone L (Jervis Bay) is questionable given that the decline was estimated from the recollections of only 1 or 2 divers prior to the year 2000.

Based on these indices, depletions in the abundance of abalone may be calculated: (i) between the commencement of the time-series (1975) and the year prior to the nominated mortality events identified for each zone; and (ii) between the year prior to the nominated mortality event (or sudden decline in abundance) and the most recent survey year (2002 for Zone L – Jervis Bay and 2003 for all other zones) (Table 3.4).

Estimated depletions of abalone prior to the nominated mortality events ranged from 0.17 – 0.67, with a mean depletion of 0.37 (Table 3.4). Thus, an estimated 63% of the biomass of abalone in 1975 was removed prior to the mortality events identified in Zones F – L of Region 1. Estimated depletions of abalone between commencement of mortality events and 2003 (or 2002 for Zone L) ranged from 0.04 – 0.35, with a mean depletion of 0.22 (Table 3.4). Thus, on average, an estimated 78% of the biomass of abalone present in the year prior to nominated mortality events, was removed (or died) by 2003.

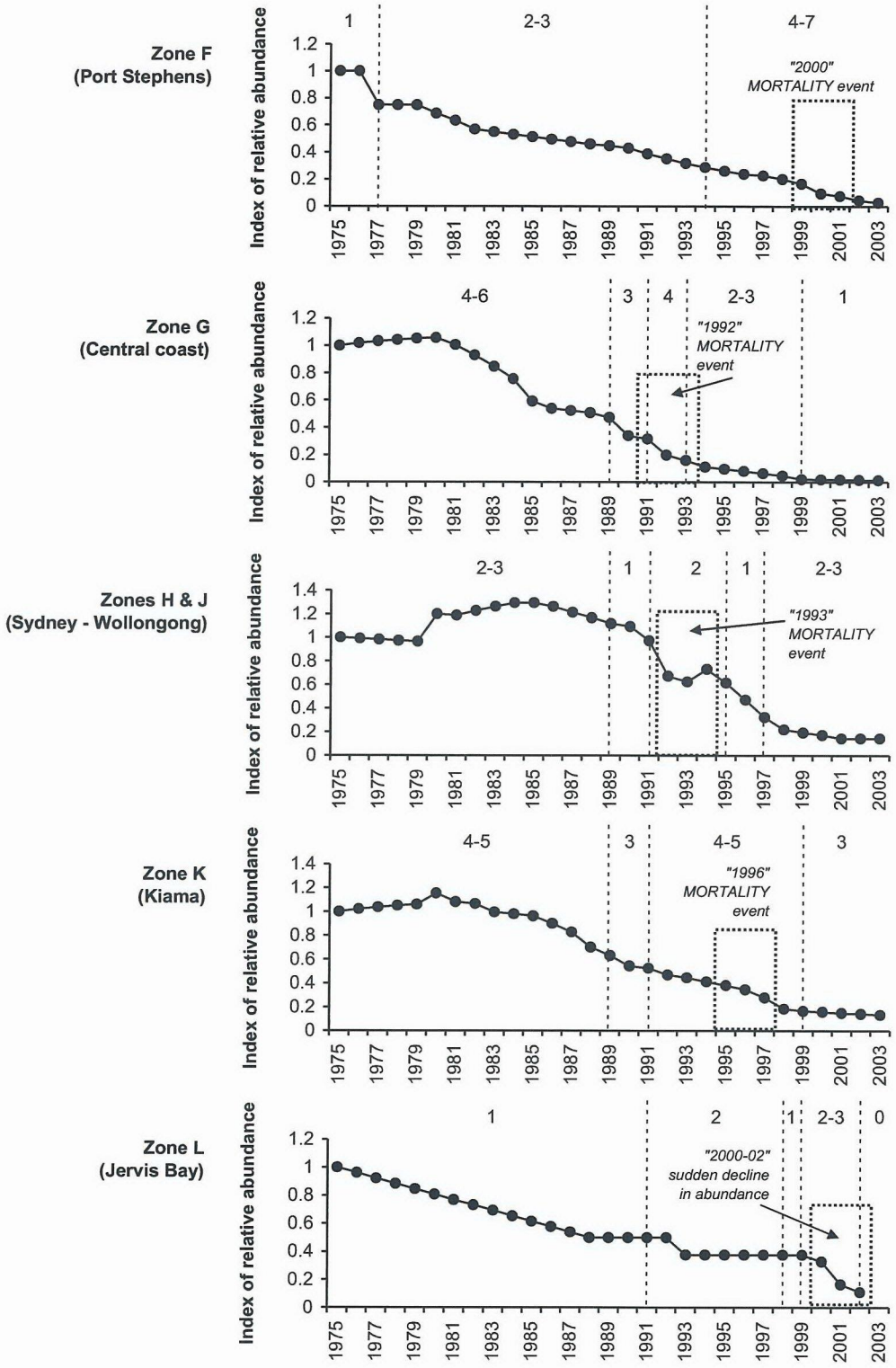

Figure 3.6. Estimated abundance of abalone, 1975 – 2003, relative to 1975, by zone, based on interviews with divers. Numbers above graphs indicate the number of estimates from individual divers that contributed to each estimate of annual change in abundance. 78% of the biomass of abalone present in the year prior to nominated mortality events, was removed (or died) by 2003.

Table 3.4. Estimated depletions of abalone, based on indices of abundance from diver interviews: prior to, during and since significant mortality events identified for zones within Region 1.

Zone	Year of "Mortality Event"	Depletion of Index PRIOR TO "mortality event"	Depletion of Index SINCE "mortality event"
Zone F Port Stephens	"2000"	$I_{1999} / I_{1975} : 0.17$	$I_{2003} / I_{1999} : 0.16$
Zone G Central Coast	"1992"	$I_{1991} / I_{1975} : 0.32$	$I_{2003} / I_{1991} : 0.04$
Zones H & J Sydney-W'gong	"1993"	$I_{1992} / I_{1975} : 0.67$	$I_{2003} / I_{1992} : 0.22$
Zone K Kiama	"1996"	$I_{1995} / I_{1975} : 0.38$	$I_{2003} / I_{1995} : 0.35$
Zone L Jervis Bay	2000 – 02 *	$I_{2000} / I_{1975} : 0.33$	$I_{2002} / I_{2000} : 0.33$
		Mean : 0.37	Mean : 0.22

** sudden decline in abundance during 2000 – 02 for Zone L, Jervis Bay – there was no observed morbidity /mortality of abalone directly observed by divers and reported during interviews.*

3.3.5. *Comparison among alternative indices of abundance and model-based estimates of biomass*

Plots of annual catches (1983 – 2002), indices of abundance from the fishery-independent survey (1994 – 2006) and indices of abundance generated from the interviews with divers on single graphics for each zone highlight the consistency between these time series (Fig. 3.7). Note that the units of the fishery-independent survey indices were first standardised to the units of the indices from diver interviews to aid graphical comparison.

For Zones F – K (Port Stephens – Kiama), the correspondence among trends in catch and the 2 other indices of abundance, for years in which the time-series overlap, is remarkable. Positive correlations between (i) "catch" and "diver interview"; (ii) "catch" and "independent survey"; and (iii) "diver interview" and "independent survey" range between 0.73 and 0.99 ($P < 0.01$ for all correlations) (Fig. 3.7). The single exception is in Zone L (Jervis Bay) where catch peaks in 2000 and 2001 against the trend in abundance estimated from interviews with divers.

The positive correlations between indices of abundance from diver interviews and the fishery-independent survey (0.98 for Zone F, Port Stephens; 0.94 for Zones H and J, Sydney-Wollongong; 0.99 for Zone K, Kiama) indicate consistency between these two indices. Therefore, the indices of abundance based on the recollections of divers during interviews could be considered reliable to the extent that they are validated against indices derived from the independent surveys.

The positive correlations between indices of abundance from diver interviews and catches suggest that changes in annual catches within Zones F – K, between 1983 and 2002, may reflect changes in abundance of abalone. This would be consistent with a scenario of serial depletion of abalone from reefs and zones within this component of the fishery.

There was remarkable consistency between 3 different estimates of depletion of abalone stocks prior to the mass mortality events identified in each zone from the early 1990's that were based on: annual catches, diver interviews and model-based stock assessments. Depletions based on annual catches, relative to 1983, averaged 0.35 (range 0.14 – 0.62 for individual zones). Depletions based on diver interviews, relative to 1975, averaged 0.37 (range 0.17 – 0.67 for individual zones). The model-based estimate of depletion of legal (> 115 mm length) biomass in Region 1 in 1998, relative to virgin, was 0.36 (90% C.I: 0.20 – 0.61) (Worthington *et al.*, 1999). Note that model-based estimates of depletion in Region 1 were also available in subsequent years up until 2000, at which time biomass of legal-size abalone was depleted to an estimated 0.18 (90% C.I: 0.09 – 0.35) of virgin biomass (Worthington *et al.*, 2001).

Estimates of depletion of abalone stocks since the mass mortality events identified in each zone from the early 1990's were available from 2 sources: fishery-independent surveys and diver interviews and these estimates were remarkably consistent. Depletions based on the fishery-independent surveys of abundance averaged 0.26 (0.23 at Port Stephens and 0.29 at Kiama). Depletions based on diver interviews 0.22 (range 0.04 – 0.35 for individual zones).

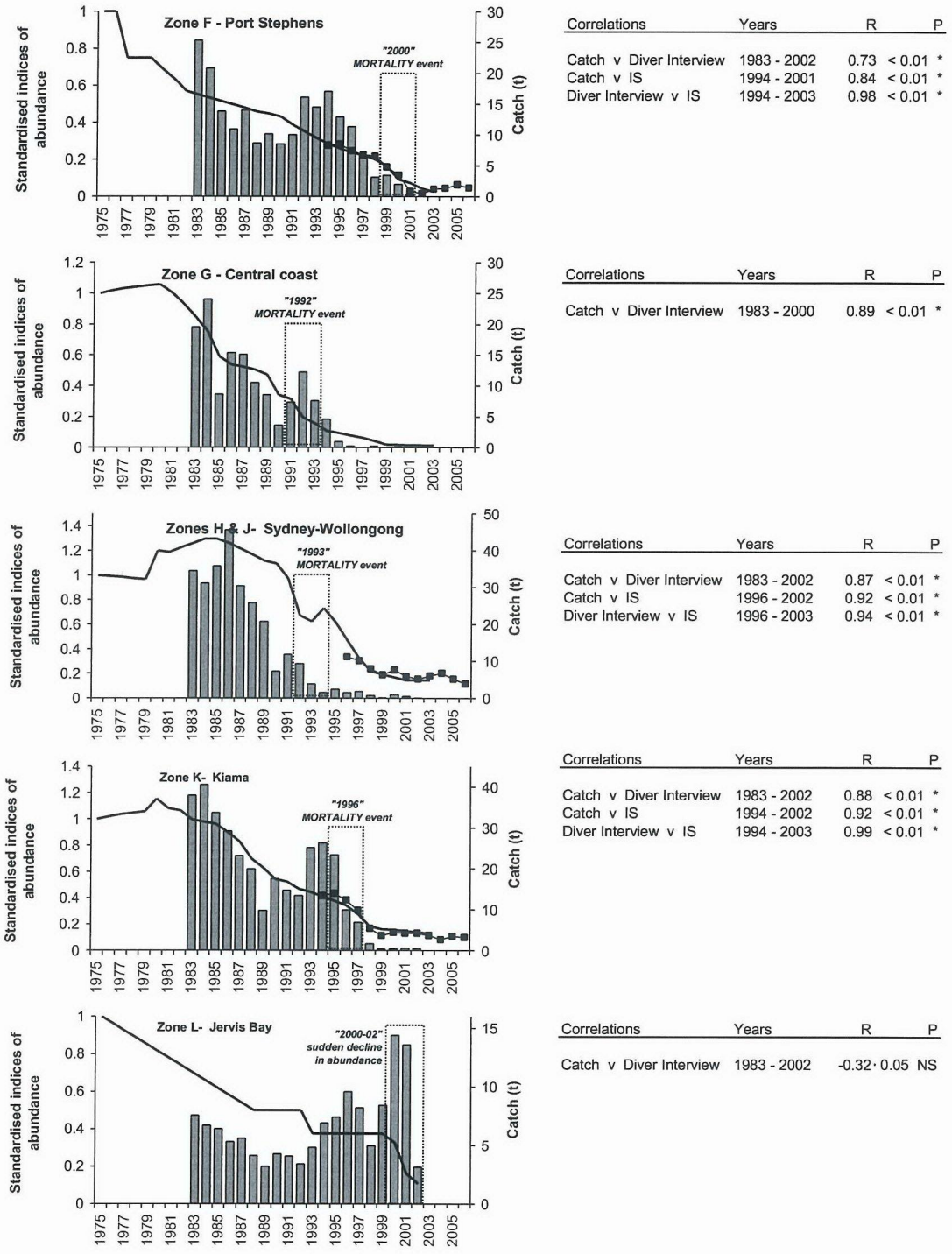

Figure 3.7. Correlations among alternative indices of abundance: catch (grey bars) and indices of abundance from the fishery-independent survey (black filled squares) and diver interviews (black line). Indices of abundance from fishery-independent survey and diver interviews have been standardised to appear on the same scale; correlation co-efficient (R) and significance of correlation (P) shown for each comparison.

3.4. Discussion

3.4.1. Identification of mortality events

Several large-scale mortality events (100's or 1000's of dead / dying abalone observed) prior to the 1990's were identified by divers during interviews (Table 3.1). On the Central Coast, 2 divers observed mortalities in the early 1980's with one associating these mortalities with a cyclone and the other diver associating mortalities, which included dead urchins and turban shells, with poor water quality from a sewage treatment outlet. Another diver recalled large-scale (1000's of dead abalone) mortalities of abalone on reefs adjacent to Minnamurra River at the time of significant flooding and consequent outflow of low-salinity water. Such observations suggest the importance of weather-driven mortality events and other factors (e.g., poor water quality) that may impact abalone stocks from time to time.

Another set of mass mortality events was observed by divers post-1990 and were associated in several instances with the presence of the protistan parasite *Perkinsus,* identified by histology by the NSW Fisheries Regional Veterinary Laboratory (Wollongbar). These mortality events were reported by multiple divers and the years in which divers observed these mortality / morbidity events within specific zones were consistent, taking into account the certainty/ambiguity that divers associated with their recollection of specific years. Thus, significant mortality events were identified in: 1992 on the Central Coast; 1993 at Sydney-Wollongong; 1996 at Kiama; and 2000 at Port Stephens. Histology detected the presence of *Perkinsus* in abalone from: the Central Coast in 1992; Sydney-Wollongong in 1993; and Kiama in 1996. No samples of abalone were collected during 2000 from Zone F (Port Stephens) during the "2000 mortality event" but a subsequent survey in 2002 detected *Perkinsus* (see Chapter 4). No observations of dead or dying abalone at Jervis Bay were made by divers who were interviewed. These divers did, however, observe sudden declines in abundance and significant numbers of empty abalone shells" during 2000 – 02. This event is referred to as the "2000 – 02 sudden decline in abundance" at Jervis Bay. Note, however, that inferences regarding *Perkinsus*-related mortality at Jervis Bay (Zone L) are confounded by the mortality due to exceptional fishing effort during 2000 and 2001 here. As for Port Stephens, *Perkinsus* was subsequently detected in abalone sampled from Jervis Bay in 2001 (see Chapter 4).

Following the observed mortality events and declines in abundance within each zone, fishing effort and therefore the presence of commercial divers decreased, eventually to zero with the total closure of Zones F – L. It is also likely that the effort of recreational divers specifically targeting abalone decreased in response to decreased abundances of abalone in the years preceding the closure. Thus, the opportunity for commercial abalone divers or recreational divers to make observations of additional mortality events in the following years was substantially reduced. Thus, there is little evidence to suggest whether or not additional mortalities occurred in the years following the initial mortality events identified by divers.

Based on the interviews of divers, we make several conclusions regarding sources of mortality for abalone during 2 periods of the fishery: (i) the period prior to; and (ii) the period following, the significant mortality events observed during the 1990's. Firstly, there was a level of "background" natural mortality that occurred during both periods and due to a variety of factors including physical disturbance due to storms, sudden drops in salinity on reefs adjacent to estuaries in flood, coastal pollution, and predation. Secondly, the effects of mortalities due to fishing on abundance of abalone were significant both prior to and since the observed mortality events in the 1990's. Finally, the significant mortality events identified during the 1990's (associated with but not necessarily due to *Perkinsus*) were, by definition, a source of mortality associated only with the second period. Thus, against a background of natural mortality and negative impacts on recruitment, declines in abundance of abalone prior to the observed mortality events in the 1990's

are attributed largely to fishing mortality and declines since the observed mortality events are attributed to a combination of fishing mortality and the observed mortality events. Note that fishing mortality includes mortality from commercial, recreational and illegal fishing.

3.4.2. Reliability of indices of abundance

There are several assumptions associated with the use of indices of abundance derived from annual catch data, counts of abalone from the fishery-independent survey and from diver interviews.

The assumption that changes in annual catch from a zone reflect changes in abundance in that zone requires careful consideration. If stocks are fully exploited and divers move their effort from one location to another once abalone have effectively been harvested (depleted) at the first location, that is, a scenario of serial depletion, then catch will crudely reflect stock abundance. Under such circumstances, catch has been used as an index of abundance in other abalone fisheries (e.g., Shepherd and Rodda, 2001). These authors also point out that declining catch is a necessary but not sufficient indicator of declining abundance. Are there reasons for declining production that are unrelated to abundance? Testimony from divers during interviews was generally that, as abalone abundance decreased in one area, they would move to other areas – consistent with the assumption. Another obvious consequence of this scenario of serial depletion is that catch rates calculated for a particular spatial unit will be maintained for that spatial unit until all productive reefs in that unit are depleted and at that point effort shifts to another spatial unit. This situation occurred in Zones F – L of Region 1 during the 1990's – the pattern of progression of effort and catch from zone to zone (Section 3.3.2 and Fig. 3.3). Declines in effort and catch in Zones G, H and J (Central coast, Sydney, Wollongong) by the early 1990s were followed by increases in effort and subsequently catches from Zones F and K (Port Stephens and Kiama) in the mid 1990s. Declines in catches from these zones in the late 1990's were followed by an increase in effort into Zone L (Jervis Bay) during 1999 – 2001. Effort and catch then declined here in 2002. Catch and effort were highly positively correlated over time in all zones and CPUE was maintained or decreased less steeply than catch and effort in each zone (Fig. 3.3).

The main issue concerning indices of abundance from the fishery-independent survey is one of potential bias that may result from the fixed survey sites not being randomly distributed in zones or across Region 1 (Fig. 3.4). Fixed sites are clustered in the northern component of Zone F, the northern component of Zone J and the southern component of Zone K.

Accuracy of indices of abundance derived from diver interviews are generally based on recollections of divers. Only in a few cases did divers access written records to inform their memories. Thus precision and bias are potentially issues here.

Despite the assumptions and uncertainties associated with the alternative indices of abundance, the significant positive correlations among the 3 series (Fig. 3.7) for all zones (excluding Zone L, Jervis Bay) provides some confidence in the conclusions drawn from these series regarding depletions of biomass.

3.4.3. Depletion of stocks prior to observed mortality events

There is no doubt that stocks of abalone declined substantially due to fishing prior to the observed mortality events in the 1990's. Depletions in Zones F – L estimated from declining catches, diver interviews and the model-based stock assessment in 1999 ranged from 0.35 – 0.37. It is also likely that estimated depletions from these three sources underestimate the depletion of stock that occurred between the 1960's and the 1990's. Depletions estimated from annual catches were relative to 1983 and those estimated from diver interviews were relative to 1975. Substantial catches came from the fishery in Region 1 prior to these years, particularly during the early 1970's. Thus, depletions relative to virgin levels of biomass would be greater. Similarly, the model-based

estimate is likely to be an underestimate. The population model that generated this estimate was calibrated using time series from the fishery–independent surveys, size-structures from commercial catches and standardised catch rates from the commercial fishery (Worthington *et al.*, 1999). There is solid evidence of serial depletion of stocks at the spatial scale of zones within Zones F – L of Region 1 and the hyper-stability of catch rates between 1983 and 1998. Calibration of the assessment model using hyper-stable catch rates would therefore lead to underestimates of depletions.

Thus, it is concluded that stocks of abalone in Zones F – L (Port Stephens – Jervis Bay) of Region 1 were depleted by more than 63% during the period prior to the mass mortality events that occurred during the 1990's.

3.4.4. Depletion of stocks since observed mortality events

It is a clear conclusion that abalone stocks in zones F – L (Port Stephens – Jervis Bay) have been massively depleted since the observed mortality events in the 1990's. Based on indices of abundance from fishery-independent surveys and derived from diver interviews, abalone biomass has been depleted by approximately 74% since the observed mortality events.

What remains unclear is the relative importance of: (i) the cause of the observed mortalities (*Perkinsus* or some other cause) and (ii) fishing mortality. Each of the mortality events identified between 1992 and 2000 in Zones F – K of the fishery and the sudden decline in abundance in Zone L at Jervis Bay (Section 3.2.1) are immediately preceded by, or are co-incident with increased catches, following a decade of decreasing catches. The "2000 mortality event" in Zone F (Port Stephens), the "1993 mortality event" in Zones H and J (Sydney-Wollongong) and the "1996 mortality event" in Zone K (Kiama) were all immediately preceded by elevated catches (Figs. 3.3 and 3.7). The "1992 mortality event" in Zone G (Central Coast) and the "2000 – 02 sudden decline in abundance" in Zone L (Jervis Bay) were both co-incident with increased catches (Figs. 3.3 and 3.7). At the time of and since the observed mortality events in each zone, four factors were contributing to declines in abundance: (i) the direct mortality due to removals of abalone in catches; (ii) the direct mortality associated with the observed mortality event (caused by *Perkinsus* or some other factor); (iii) decreased recruitment to the stock compared to previous years due to decreases in abundance of mature stock resulting from "i" and (iv) decreased recruitment to the stock compared to previous years due to decreases in abundance of mature stock resulting from "ii".

Compromised recruitment following significant depletion of mature stock is well described for abalone fisheries (Prince *et al.*, 1988; Hobday *et al.*, 2001; Miner *et al.*, 2006). As dioecious broadcast spawners, aggregation of adult abalone at less than some critical density may significantly compromise the probability of successful fertilisation (Prince *et al.*, 1987; Miller and Lawrenz-Miller, 1993). Moreover, evidence suggests abalone larvae have limited dispersal and therefore localised recruitment (McShane *et al.*, 1988; Prince *et al.*, 1988; Hamm and Burton, 2000; Chambers *et al.*, 2006; Miner *et al.*, 2006). The estimated 63% depletion of stocks in Zones F – L of Region 1 prior to observed mortality events, followed by a further 74% depletion following the observed mortalities suggests that current depletions may be less than 10% of virgin levels. If the current biomass of abalone is indeed an order of magnitude less than pre-exploitation biomass and the generalisations about limited larval dispersal and localised recruitment are applicable in this fishery, then recovery of stocks is likely to be a slow process.

Fishery-dependent catch and effort data has not been available since November 2002, due to the closure of Zones F – L within Region 1. Moreover, the fishery-independent survey was discontinued after 2007. The need for additional information about abundances of abalone in this component of Region 1 is discussed in Chapter 7.

3.5. Conclusions

- Mass mortalities of blacklip abalone, observed by divers, occurred during the year (+/- 1 year): 1992 on the Central Coast, 1993 at Sydney-Wollongong, 1996 at Kiama and 2000 at Port Stephens. A sudden decline in abundance of abalone, unaccompanied by observations of morbidities or mortalities, occurred at Jervis Bay during 2000 – 02.

- Stocks of abalone between Port Stephens and Jervis Bay were significantly depleted by at least 63%, due to fishing, prior to the documented mortality events in the 1990's.

- Stocks of abalone between Port Stephens and Jervis Bay were depleted by approximately 74% due to a combination of the documented mortality events during the 1990's and limited fishing.

- The estimated 63% depletion of stocks prior to, and 74% depletion since the documented mortality events, suggests that the stock of abalone between Port Stephens and Jervis Bay may have been depleted to less than 10% of virgin abundance.

- Given limited larval dispersal and localised recruitment in abalone populations, the recovery of stocks of blacklip abalone between Port Stephens and Jervis Bay is likely to be a slow process.

4. IDENTIFICATION AND GEOGRAPHIC DISTRIBUTION OF THE PARASITE PERKINSUS IN ABALONE IN NSW

4.1. Introduction

4.1.1. Species and life history

The genus *Perkinsus* includes several species of protistan parasites that infect many species of marine mollusc throughout the world, some of which have been associated with mass mortalities (Villalba *et al.*, 2004). Taxonomic and phylogenetic placement of the genus *Perkinsus* continues to be debated. Levine (1978), having established the genus *Perkinsus*, placed it in Phylum Apicomplexa. More recent studies of taxonomic affiliations of *Perkinsus*, based on DNA sequencing technology, have suggested a closer affiliation with the dinoflagellates than the apicomplexans within a group of taxa referred to as the alveolates which includes ciliates, apicomplexans and dinoflagellates (Cavalier-Smith, 1993). A new phylum, Perkinsozoa, to include *Perkinsus* and other closely related taxa has even been proposed (Noren *et al.*, 1999). However, studies have continued to provide conflicting evidence regarding taxonomic placement of *Perkinsus* and there remains no generally accepted conclusion (Villalba *et al.*, 2004). There are 5 species of *Perkinsus* currently considered valid, *P. marinus*, *P. olseni* (junior synonym *P. atlanticus*), *P. qugwadi*, *P. chesapeaki* (and conspecific *P. andrewsi*) and *P. mediterraneus* (Villalba *et al.*, 2004; Burreson *et al.*, 2005). Recently, 2 new species, *P. beihaiensis* and *P. honshuensis* have been proposed (Dungan and Reece, 2006; Moss *et al.*, 2008).

The life cycle of *Perkinsus* species consists of 3 main life-stages (Fig. 4.1). The following description is based on the terminology and descriptions of Villalba *et al.* (2004) and Goggin and Lester (1995). The trophozoite occurs in the tissues of the live host. Vegetative proliferation within the host involves successive bipartitioning of the trophozoite to yield up to 32 immature trophozoites (or merozoites) contained in a wall, this multicellular form being referred to as the tomont or schizont. Upon wall rupture, the immature trophozoites are liberated and subsequently become mature trophozoites (also referred to as meronts). When infected host tissues are placed in Ray's fluid thioglycollate medium (RFTM), trophozoites enlarge and develop a thick wall becoming a hypnospore (also referred to as prezoosporangium). It is postulated that this may also occur naturally in the tissue of moribund hosts and in anaerobic environments such as the gut or in faeces and pseudofaeces within or external to the live host (see Bushek *et al.*, 2002; Ragone Calvo *et al.*, 2003b). In seawater, the hypnospore develops into a zoosporangium containing thousands of zoospores. Subsequently, these motile, biflagellated zoospores are released into the seawater through one or 2 discharge tubules. Knowledge of the life cycle is incomplete. Under experimental conditions, trophozoites free of host tissue can also survive in seawater and infect a susceptible host (Chu and Lund, 2006). Whilst all three life-stages have been shown to cause infections in shellfish (mostly oysters) under experimental conditions (Villalba *et al.*, 2004), the principal stage for transmission of the disease in the natural environment is unknown (Chu, 1996).

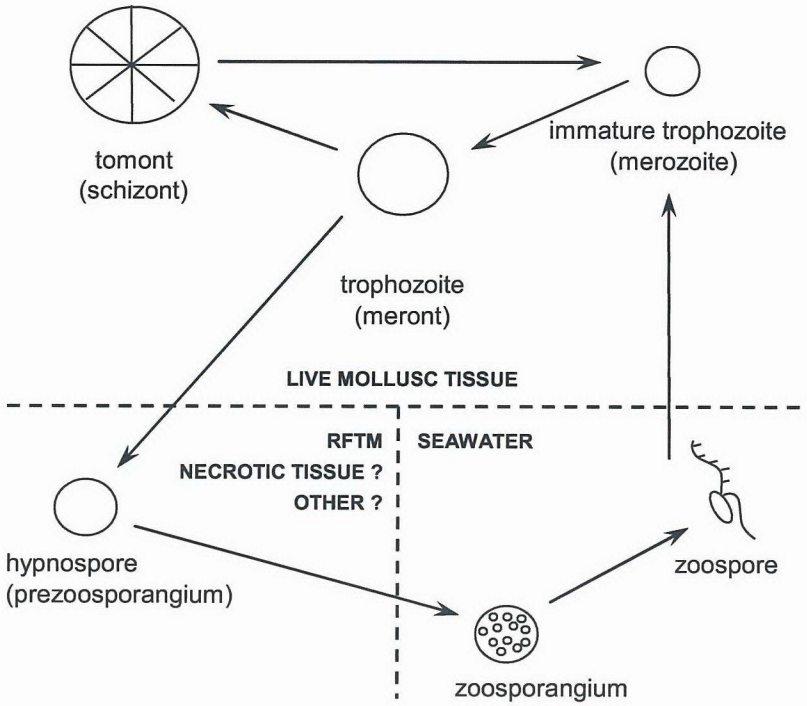

Figure 4.1. Life cycle of *Perkinsus* spp. Adapted from Goggin and Lester (1995), based on description by Villalba *et al.* (2004).

Perkinsus sp. has been identified in numerous mollusc species in Australia. *Perkinsus* has been widespread in bivalve species in Queensland (Goggin and Lester, 1987), northern Western Australia (Hine and Thorne, 2000) and South Australia (Goggin and Lester 1995). *Perkinsus* sp. has also been identified in 4 species of abalone (greenlip abalone *(H. laevigata)*, blacklip abalone (*H. rubra*), *H. cyclobates* and *H. scalaris*) in South Australia (Lester *et al.*, 1990; Goggin and Lester 1995), blacklip abalone in NSW (Callinan and Landos, 2006) and in a single instance on the gills of an abalone in Western Australia (Handlinger *et al.*, 2006).

The original description of *P. olseni* was based on microscopic observation and culture of *Perkinsus* found in blacklip abalone in South Australia (Lester and Davis, 1981). Subsequently, molecular studies confirmed *P. olseni* as the species also infecting greenlip abalone from the same location and bivalves *Anadara trapezia* and *Chama pacificus* from Queensland and the Great Barrier Reef (Goggin, 1994; Goggin and Lester, 1995; Murrell *et al.*, 2002). *P. olseni* and *P. atlanticus,* conspecific based on DNA sequencing (Murrell *et al.*, 2002), are also distributed in a variety of gastropod and bivalve hosts in New Zealand (Dungan *et al.*, 2007), Asia (Leethochavalit *et al.*, 2003; Park *et al.*, 2005, 2006b), Portugal (Azevedo, 1989), Spain (Casas *et al.*, 2002; Elandaloussi *et al.*, 2009) and Uruguay (Cremonte *et al.*, 2005). *Perkinsus* species have also been reported from France and Italy but have yet to be positively identified as *P. olseni* (Da Ros and Canzonier, 1985; Goggin, 1992).

Whilst *Perkinsus* sp. infections have been identified in blacklip abalone in NSW, *P. olseni* has not been specifically identified. However, given the identification of *P. olseni* infecting abalone in South Australia, it has been assumed by many and in various reports (e.g., Andrew *et al.*, 1996; NACA, 2004) that the species infecting blacklip abalone in NSW is indeed *P. olseni*. Based on apparent variations in pathogenicity of *Perkinsus*, observed morphological differences and genetic variation among isolates, it has been suggested that there may be multiple strains or more than one

species of *Perkinsus* infecting molluscs in Australia (Dungan and Roberson, 1993; Goggin and Lester, 1995; Hine and Thorne, 2000). Thus, it should not be assumed that the *Perkinsus* species infecting blacklip abalone in NSW is *P. olseni*. Identification by PCR, DNA probes (*in situ*) or DNA sequencing (recommended method, OIE, 2009), is necessary.

4.1.2. Perkinsus in NSW and history of surveys

Fundamental to investigating *Perkinsus*-related mortalities of abalone in NSW are issues concerning the identification of this parasite in abalone and variations in the prevalence of *Perkinsus* infections in abalone both spatially (geographic range and at finer spatial scales) and temporally among years.

Following observed mortalities and localised declines of abalone stocks between the Central Coast and Kiama during the 1990's, *Perkinsus* was first identified in abalone in NSW. Histological examination of 2 dead abalone collected during the "1992 mortality event" on the Central Coast at Terrigal revealed *Perkinsus* infections (Appendix 4.1 in Section 12.4.1 and Table 4.1). Similarly, samples of abalone collected immediately following the "1993 mortality event" at Sydney-Wollongong and the "1996 mortality event" at Kiama also tested positive to *Perkinsus* by histology (Appendix 4 in Section 12.4 and Table 4.1). Subsequent to these initial observations, samples of abalone were collected from several locations for histological examination but sample sizes were small, few sites were surveyed and these sites were mostly within areas where mortalities had been observed (Table 4.1). In 2001, a total of 200 abalone were collected from Kiama and Jervis Bay by NSW Fisheries and *Perkinsus* infections were detected at both locations. Thus, *Perkinsus* was detected for the first time at Jervis Bay (North Jervis Bay) in 2001 (Table 4.1). The broader geographic distribution of *Perkinsus* in abalone along the NSW coast was not examined until 2002 and 2003 when complementary surveys by NSW Fisheries and the NSW component of the National Survey of Diseases in Abalone (Callinan and Landos, 2006; Handlinger *et al.*, 2006) were completed. The geographic range of the survey done in 2002 by NSW Fisheries was between Crowdy Head in the north and Pretty Beach (to the north of Bateman's Bay) on the south coast of NSW. All abalone sampled were tested using Ray's fluid thioglycollate assay on gill tissue and histology was examined for a sub-set of abalone collected as part of the NSW component of the National Survey of Diseases in Abalone (hereafter, NSDA). The latter study also examined the histology of additional abalone collected in 2003 between South Bateman's Bay and Eden (Table 4.1).

The 2002-03 surveys detected *Perkinsus* infecting abalone between Port Stephens and Jervis Bay, corresponding to the zones within Region 1 of the fishery in which mortality events or sudden declines in abundance had been reported by divers during the 1990's and early 2000's. Note that the 2002 survey included limited sampling to north of Port Stephens (only as far north as Crowdy Head) and extended no further south than North Bateman's Bay. Thus, the presence of *Perkinsus* infections in abalone further north and south on the coast of NSW was not examined. Whilst the supplementary sampling for the NSDA in 2003 did extend sampling to the south as far as Eden, histology and not Ray's test was used to indicate the presence of *Perkinsus* in these samples. It is unfortunate that the 2002-03 surveys occurred after the mortality events identified in Chapter 3. Nevertheless, these data provide the only broad-scale survey that can be used as a baseline for comparison with data collected during the present study in which the geographic range of sampling is extended further to the north and south of NSW. Thus, there was not only a clear need to update (since 2002) the geographic range of *Perkinsus* infections in abalone on the NSW coast, but to also extend the range of the survey.

Table 4.1. Previous surveys of the prevalence of Perkinsus in abalone and other mollusc species on the NSW coast. Shaded rows indicate the first year in which *Perkinsus* were detected at a location. * indicates that a sub-sample of these abalone (from the 2002 survey by NSW Fisheries) were examined using histology as part of the NSW component of National Survey of Diseases in Abalone (Callinan and Landos, 2006; Handlinger *et al.*, 2006). Number of infections reported in these instances refers to results from Ray's test.

Location / Area	Year	Month	Species	Sampling			*Perkinsus* +		Reference
				Sites	N	Method	Sites	n	
Crowdy Head - Forster									
Crowdy Head to Seal Rocks	2002	May	*H. rubra*	6	120	Ray's + Histo*	0	0	NSW Fisheries 2002 survey; Histo: Callinan & Landos (2006)
Port Stephens - Broughton Is.									
Broughton Is to Fisherman's Bay	2002	Feb-May	*H. rubra*	5	101	Ray's + Histo*	2	6*	NSW Fisheries 2002 survey; Histo: Callinan & Landos (2006)
Yaccaba Head and Shark Is	2003	Apr	*H. rubra*	2	40	Ray's	0	0	Gill (2003)
Wanda Wanda Head to Stockton	2003	Apr	Other spp.	5	190	Ray's	0	0	Gill (2003)
Sunny Corner	2004	Nov	*H. rubra*	1	11	Ray's	1	2	NSW Fisheries 2004 site sample
Sunny Corner	2004	Nov	Other spp.	1	8	Ray's	0	0	NSW Fisheries 2004 site sample
Central Coast & Sydney									
Terrigal (Skillion)	1992	May	*H. rubra*	1	2	Histology	1	2	NSW Fish. Vet. Lab. report WN92/1337 (dead abalone)
Coalcliff	1993	June	*H. rubra*	1	4	Histology	1	1	NSW Fish. Vet. Lab. report WN93/1341 (assoc. mort. event)
Swansea	1995	Nov	*H. rubra*	1	1	Histology	1	1	NSW Fish. Vet. Lab. report WN95/3672
Forresters to Curracurang	2002	Feb-May	*H. rubra*	5	100	Ray's + Histo*	2	4*	NSW Fisheries 2002 survey; Histo: Callinan & Landos (2006)
Forresters to Skillion	2004	Apr	*H. rubra*	3	76	Ray's	1	2	NSW Fisheries 2004 site samples
Kiama									
Easts Beach to Gerringong	1996	Apr	*H. rubra*	7	13	Histology	6	10	NSW Fish. Vet. Lab. report WN96/1001 (assoc. mort. event)
Kiama Blowhole to Black Head	1996	May	*H. rubra*	3	30	Histology	1	1	NSW Fish. Vet. Lab. report WN96/1440 (assoc. mort. event)
Kiama Blowhole to Black Head	2001	Mar	*H. rubra*	5	100	Ray's	2	2	NSW-Fish. 2001 Kiama & Jervis Bay survey
Bass Point to Gerroa	2002	Apr	*H. rubra*	4	79	Ray's + Histo*	2	2*	NSW Fisheries 2002 survey; Histo: Callinan & Landos (2006)
North Jervis Bay									
Currarong	1996	May	*H. rubra*	1	10	Histology	0	0	NSW Fish. Vet. Lab. report WN96/1440
Drumsticks to Pt Perpendicular	2001	May	*H. rubra*	3	75	Ray's	1	3	NSW Fish. 2001 Kiama & Jervis Bay survey
Beecroft Head to Crocodile Head	2002	May	*H. rubra*	5	100	Ray's	2	13	NSW Fisheries 2002 survey
Drum & Drumsticks Is & Bay	2003	Jun-Jul	*H. rubra*	2	30	Ray's	1	2	Gill (2003)
Drum & Drumsticks Is	2003	Jul	Other spp.	1	7	Ray's	0	0	Gill (2003)
South Jervis Bay									
Bowen Is	2001	May	*H. rubra*	1	25	Ray's	0	0	NSW Fish. 2001 Kiama & Jervis Bay survey
Bowen Is to St Georges Hd	2002	Apr-May	*H. rubra*	5	100	Ray's + Histo*	4	19*	NSW Fish. 2002 survey; Histo: Callinan & Landos (2006)
Bowen Is	2003	Jun	*H. rubra*	1	21	Ray's	0	0	Gill (2003)
St Georges Hd	2003	Jun	*H. rubra*	1	24	Ray's	0	0	Gill (2003)

Table 4.1. (cont'd)

Location / Area	Year	Month	Species	Sampling			*Perkinsus* +		Reference
				Sites	N	Method	Sites	n	
North Ulladulla									
Sussex to Ulladulla lighthouse	2002	Apr	*H. rubra*	5	100	Ray's + Histo*	0	0	NSW Fisheries 2002 survey; Histo: Callinan & Landos (2006)
Sussex & Bendalong	2003	Jun	*H. rubra*	2	43	Ray's	0	0	Gill (2003)
St Georges Basin to Burril Lake	2003	Jun	Other spp.	6	152	Ray's	0	0	Gill (2003)
North Bateman's Bay									
Brush Is.	1996	May	*H. rubra*	1	10	Histology	0	0	NSW Fish. Vet. Lab. report WN96/1440
Nugen to Doyles	2002	Apr	*H. rubra*	5	102	Ray's + Histo*	0	0	NSW Fisheries 2002 survey; Histo: Callinan & Landos (2006)
South Bateman's Bay									
Richmond & Mossy Pt	2003	Mar	*H. rubra*	2	49	Histology	0	0	Callinan & Landos (2006)
Narooma									
Narooma & Mystery Bay	2003	Mar	*H. rubra*	2	47	Histology	0	0	Callinan & Landos (2006)
Tathra - Merimbula									
Tathra & Merimbula	2003	Mar	*H. rubra*	2	75	Histology	0	0	Callinan & Landos (2006)
Eden									
Eden & Disaster Bay	2003	Mar	*H. rubra*	2	55	Histology	0	0	Callinan & Landos (2006)

4.1.3. Diagnostic tests

The specificity and sensitivity of a diagnostic test will affect its capacity to distinguish between *Perkinsus*-like organisms, organisms of the genus *Perkinsus* and positively identify species within the genus *Perkinsus*. Specificity (i.e., the range of organisms and cell types resulting in positive tests) and sensitivity (the smallest quantity that can be reliably detected) of tests used for the detection of *Perkinsus* spp. in hosts vary among the types of test used and also according to the way in which each test is applied (e.g., which tissues/organs, quantity of tissue examined) (Bushek *et al.*, 1994; Villalba *et al.*, 2004). Obviously, understanding these aspects of test performance is vital to the interpretation of positive and negative test results. The standard method for diagnosing *Perkinsus* infections is Ray's test in which host tissues are incubated in Ray's fluid thyoglycollate medium (RFTM) and live trophozoites enlarge to form hypnospores which stain blue-black with Lugol's iodine (reviewed in Villalba *et al.*, 2004). Histological examination of tissues is also routinely used for observing and therefore detecting *Perkinsus* trophozoites and tomonts as well as associated pathology. Various immunological and molecular diagnostic techniques have been developed during the past decade (Villalba *et al.*, 2004), most recently polymerase chain reaction (PCR) assays that target individual *Perkinsus* species: *P. marinus* (e.g., Robledo *et al.*, 1998; Yarnall *et al.*, 2000); *P. olseni* (Robledo *et al.*, 2000; Murrell *et al.*, 2002; Park *et al.*, 2005).

There are no direct comparisons of the sensitivity and specificity of the 3 main types of test (Ray's, histology, PCR) done "simultaneously" on the same batch of hosts. Comparisons have, however, been made between pairs of tests. Ray's test has generally found to be more sensitive than histology for detection of *Perkinsus*, especially for light infections (e.g., McLaughlin and Faisal, 1999; Almeida *et al.*, 1999; Villalba *et al.*, 2005). Ray's test on haemolymph has been used as a means of non-lethal sampling (Gauthier and Fisher, 1990) but was reported to be a less sensitive method than Ray's test on tissue (Rodriguez and Navas, 1995). The whole body of hosts (oyster: Fisher and Oliver, 1996; carpet shell clam: Almeida *et al.*, 1999) has been incubated and included in modified assays based on Ray's test. Due to differential infection of host organs by *Perkinsus*, this technique offers increased sensitivity when compared to Ray's test on single tissues (e.g., Bushek *et al.*, 1994; Rodriguez and Navas, 1995; Villalba *et al.*, 2005). In particular, the intensity of infections can be more accurately determined (Bushek *et al.*, 1994; Park *et al.*, 2006b). However, due to the volume of tissue present in large molluscs, the application of the whole body burden assay to blacklip abalone is problematic. Further, specificity of Ray's test is an issue given that it can give false positives for other organisms such as dinoflagellates (Almeida *et al.*, 1999) and *Perkinsus*-like organisms such as *Pseudoperkinsus tapetis* (Novoa *et al.*, 2002). PCR assays developed for *P. marinus* (Robledo *et al.*, 1998; Yarnall *et al.*, 2000) were found to be more specific and sensitive than Ray's test on individual tissues, although incubation of the whole host body gave comparable results for sensitivity (Yarnall *et al.*, 2000). In summary, Ray's test and PCR are generally considered to be more sensitive than histology. The World Organisation for Animal Health (OIE) notes that the use of histology for the purpose of targeted surveillance of *Perkinsus* has application in some situations but recommends Ray's test on tissue for surveillance of the parasite in abalone and confirmation of *P. olseni* by PCR, DNA probes (*in situ*) or gene sequencing (OIE, 2009).

4.1.4. Terminology

Within this study, several diagnostic methods (Ray's test, histology, PCR and gene sequencing) were used to test for the presence of *Perkinsus* in abalone (described in detail in Section 4.2.1.3). Because the specificity of these methods differ, we adopt the pedantic approach in this chapter of referring to positive results diagnosed: by Ray's test as cases of suspected-*Perkinsus* sp.; by histology as cases of *Perkinsus* sp.; by *P. olseni*-specific PCR and/or gene sequencing as cases of *P. olseni*; and diagnosed by gene sequencing of PCR products as a previously unidentified species of *Perkinsus* or variant strain of *P. olseni* as *Perkinsus* sp.-variant ITS.

4.1.5. 2005 survey of Perkinsus

The present study used a combination of Ray's test on gill tissue, histology of multiple organ tissues and PCR on gill and mantle tissue to identify suspected-*Perkinsus, Perkinsus* sp. or *P. olseni* in abalone sampled during a broad-scale survey of the distribution and prevalence of *Perkinsus* infections in blacklip abalone at multiple sites within multiple locations along the NSW coast. Associated with this broad-scale survey were 2 auxiliary surveys done in the vicinity of Jervis Bay, the presumed "southern front" of infection: (i) a pilot survey to assess the prevalence and intensity of *Perkinsus* infections at a finer spatial scale at a single site; and (ii) a survey done at 4 sites to assess differences in the prevalence of infections between February – March and April – May. Correspondence of results of Ray's, histology and PCR tests was also examined to aid our interpretation of the likely distribution of *Perkinsus* within NSW where abalone populations occur.

Thus, the principal objectives of this component of the project, presented in this chapter, were to:

(i) Survey the geographic distribution of *Perkinsus* in abalone (in 2005) and extend the geographic range covered by the 2002-03 surveys;

(ii) Determine the spatial variability of prevalence and intensity of *Perkinsus* infections in blacklip abalone (at spatial scales of: locations on the coast; sites within locations; areas adjacent to sites) and compare with results from 2002-03 surveys;

(iii) Assess the correspondence of positive and negative test results among 3 alternative diagnostic tests for the presence of *Perkinsus*: Ray's test, histology and PCR;

(iv) Positively identify the species of *Perkinsus* infecting abalone in NSW;

4.2. Methods

4.2.1. Survey of the spatial distribution, prevalence and intensity of Perkinsus infections in abalone on the NSW coast

4.2.1.1. Survey designs and sampling methods

The survey extended from Bonnie Hills on the north coast to Wonboyn on the far south coast of NSW. Between 4 and 6 sites were sampled within each of 12 locations (Fig. 4.2). For the component of the 2005 survey that included the geographic range of the 2002 survey (9 locations between Crowdy Head and North Bateman's Bay), sites sampled during the 2002 survey were included in the 2005 survey. At 5 of the sites sampled in 2002, no abalone could be located in 2005 and we attempted to add replacement sites to the 2005 survey. Replacement sites were sampled in 4 of these 5 instances. The geographic range of the 2005 survey extended a distance of 40 km further to the north of the 2002 survey, including an additional location ("North Coast"). The 2005 survey also extended approximately 200 km further south than the 2002 survey, including an additional 2 locations (South Bateman's Bay and Tathra/Eden). Distances between locations ranged between 10 and 85 km on the north coast and 5 and 30 km on the south coast where suitable rocky reefs and populations of abalone are closer to one another. The depth at which abalone were sampled ranged from 2 – 10 m.

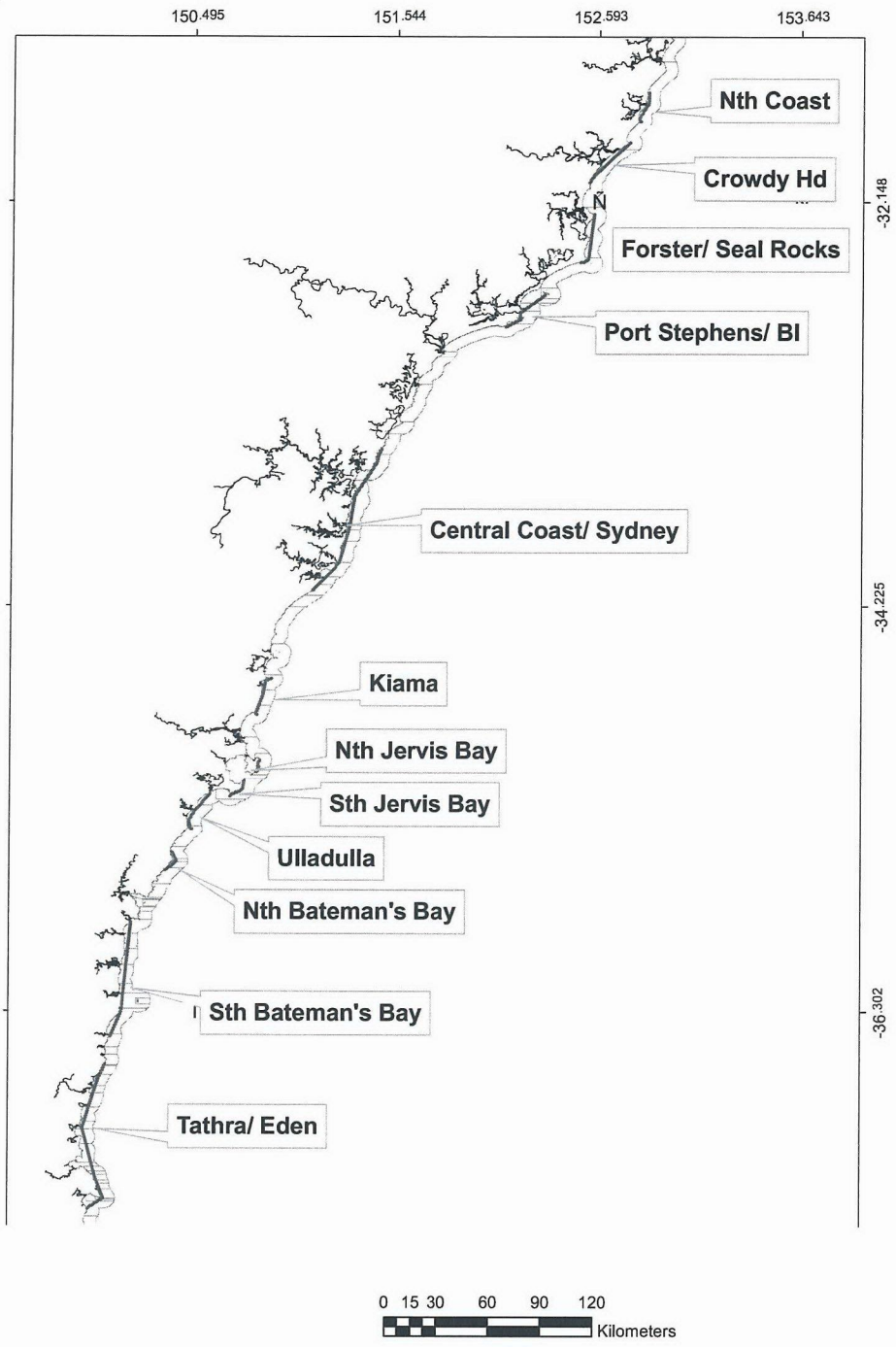

Figure 4.2. Map of NSW coast showing 12 locations where abalone were sampled for broad-scale study. Brown lines denote the range of survey sites within each location.

To increase the chance of detecting *Perkinsus* within abalone the survey was done during Austral summer and autumn, when warmer water temperatures are likely to favour parasite proliferation (discussed in Chapters 5 and 6). The survey was completed between January and May, 2005.

A target sample size of 20 abalone per site and therefore 100 abalone per location (5 sites) was selected on the basis that: (i) this corresponded with the sample size used in the 2002 broad-scale survey and (ii) this sampling intensity provided a ≥ 95% probability of detecting at least 1 abalone infected with *Perkinsus*, if 3% of the population at a location were infected (assuming 100% test sensitivity). At the scale of sites, a sample size of 20 provided only a ≥ 46% probability of detecting at least 1 abalone infected with *Perkinsus*, if 3% of the population at a site were infected. Obviously, for any given sample size, the probability that the absence of *Perkinsus* in abalone sampled from a site or location accurately indicates the absence of *Perkinsus* at that site or location depends on the prevalence of *Perkinsus* infections in abalone at that site or location. For example, if 14% of abalone were infected with *Perkinsus*, then a sample of size of 20 (the sample size at each site in the 2005 survey) would provide 95% probability of detecting at least 1 positive test result.

A total of 1190 abalone, with shell lengths (SL) ranging between 35 mm and 145 mm (approximately half were sub-legal, < 115 mm SL) were collected by scuba divers between January and early May 2005. All abalone were alive and no moribund abalone were observed. At each of 59 sites, an average of 20 abalone (minimum = 19, maximum = 22) representing a mixture of legal and sub-legal sizes were collected. As far as possible, abalone were collected randomly from the area searched by the diver. The proportion of abalone sampled at each location that were of sub-legal size (< 115 mm SL) ranged from 25% to 75%, but was generally between 40% and 60%.

To test for a bias in results due to timing of sampling (i.e., early or late in the season), populations of abalone at four sites that were sampled in February were re-sampled during April 2005 (see Section 4.2.2.2). Our main concern was that there was consistency in results between February and April sampling times for "negative" sites, where *Perkinsus* was not detected in abalone sampled. We restricted our sampling to the Jervis Bay area, where *Perkinsus* infections were most prevalent.

In the field, abalone from each site were kept alive and separated, by placing them in foam inserts which were stacked and stored within insulated containers or commercial abalone bins that were kept cool. No water was added to the containers or bins, thereby reducing the risk of cross contamination among abalone.

4.2.1.2. Data collection and tissue biopsies

Processing of abalone occurred within 24 hr of collection. All abalone were examined for gross pathological abnormalities at the time of processing. The shell of each abalone was cleaned of epiphytic material and a measurement of the shell length (along longest axis of shell) to the nearest mm was recorded. Abalone were shucked and the sex of each mature abalone was also determined.

For Ray's test, approximately 2 cm of gill tissue was excised from each individual (using sterile instruments) and placed into sterile tubes containing 20 ml of Ray's fluid thioglycollate medium (RFTM). To prohibit bacterial and fungal growth during cultivation, 200 units of Mycostatin (nystatin) and 2 mg of Chloromycetin (chloramphenicol) dissolved in 50 µl of distilled water were added to each tube (Ray, 1952, 1966). A further sample of gill and mantle (~ 1 cm or 200 mg total tissue) was excised from each abalone, placed in a sterile container, and archived (frozen at -80°C) for subsequent PCR analysis. The remainder of the abalone was preserved in 10% formalin in seawater for histological examination. To aid with preservation the foot of each abalone was scored to a depth of ~ 0.5 cm at ~ 2 cm intervals.

4.2.1.3. Diagnostic methods

Ray's test

Gill tissue from all abalone (with one exception) that were collected was tested for presence of *Perkinsus* sp. parasites using Ray's test. Each ~ 2 cm piece of gill tissue was incubated in RFTM fortified with antibiotics at temperatures between 22 – 25°C for 5 – 7 days, in the dark. It was assumed that all known life stages of viable *Perkinsus* cells would enlarge to the hypnospore (pre-zoosporangia) stage during incubation (Bushek *et al.* 1994). After incubation the gill tissue was removed using sterile forceps and placed into a clean Petri dish and examined under a dissection microscope at 25 – 40x magnification to identify dark particulates, which could be confused with stained *Perkinsus* cells. Two drops of 20 – 33% (v/v) Lugol's iodine were then added to the tissue and after ~10 mins the tissue was examined again under magnification for presence of *Perkinsus* hypnospores, which appear as spherical cells of diameter 50 – 70 µm with their cell walls stained blue-black (Ray 1952; and see examples in Appendix 5 – Section 12.5.1). The total number of hypnospores in the entire preparation was estimated and the level of infection was graded according to a modified version of the Mackin scale (Ray, 1954), with a numerical range between 0 and 5 as follows: 0 = uninfected; 0.5 = less than 11 parasites found in entire tissue preparation (i.e., in the gill section); 1 = 11 – 100 cells present in entire preparation; 2 = >100 cells, some areas free of parasites but other areas show localised concentrations of 25 – 50 cells; 3 = >100 cells, localised concentrations of 50 or more cells and some areas free of parasites; 4 = parasite cells present in large numbers (>> 100 cells), but less than half of tissue covered macroscopically; 5 = enormous numbers of parasite cells, most of the tissue is covered macroscopically. Due to the subjective nature of the scale the grading for each sample was initially done by two persons to ensure consistency of results between the 2 examiners. Positive diagnoses from Ray's test in a sub-set of tissue samples (n = 8) were confirmed by a third researcher (C. Hayward) from South Australia.

Histological examination

A total of 209 abalone were examined using histological methods. For each site, histology was examined for all abalone which were positive by Ray's test and at least the equivalent number of randomly chosen abalone that were negative by Ray's test. If all abalone from a site were negative by Ray's test then 1 or 2 abalone were randomly chosen for histology. At two sites outside of the previously documented range for *Perkinsus* in NSW (Diamond Head Bay and Merimbula) where small numbers of abalone tested positive to *Perkinsus* by Ray's test and were graded with low-level infections, histology was examined for all abalone.

Five standardised partial cross-sections of tissue were taken from whole abalone. All sections were ~5 mm in thickness and cut to the depth of the foot muscle. The first two sections were cut at the anterior end and midway along the gills respectively. The third section was cut posterior to the gills and included the pericardial cavity. The fourth section was cut at the most posterior aspect of the abalone, opposite the mouth. The fifth section was cut approximately midway along the right foot muscle. The aim of histological screening was to include foot muscle, epipodium, oesophagus (where possible), digestive gland and hind-gut, kidney, heart (where possible), gills, and gonad from each individual. The tissue samples were placed in labelled histological cassettes, and fixed immediately in 10% formalin. After at least 24 hrs fixation, samples were processed through alcohol, embedded in paraffin blocks, and sectioned (2 – 5 µm thickness) and stained with haematoxylin and eosin using standard laboratory methods.

Stained tissue sections were examined for the presence of parasites consistent in size and morphological appearance with *Perkinsus* (with reference to positive controls archived from the

National Disease Survey) and for other tissue abnormalities using light microscopy. For each abalone examined, the presence or absence of *Perkinsus* within organs and tissues was recorded and graded as 0 (no cells), 1 (1 – 5 cells), 2 (> 5 to 10 cells) or 3 (> 10 cells). For the purposes of this study, *Perkinsus* trophozoites and other life stages were not differentiated (see also Chapter 5). All histological examinations were done by P. Gill at the DPI Regional Veterinary Laboratory, Wollongbar.

PCR assay

A partial tissue sample of ~100 mg from each of 40 archived gill and mantle tissues was sent to the OIE reference laboratory at the Virginia Institute of Marine Science (VIMS), USA, for PCR analysis and gene sequencing to detect the presence of *Perkinsus* and identify the species. Samples to undergo traditional PCR testing were selected from abalone that had been tested by both Ray's test and histology to include 32 *Perkinsus*-positive and suspected-*Perkinsus* cases (low- and high-grade infections determined by Ray's test) and 8 *Perkinsus*-negative samples. The 32 *Perkinsus*-positive samples were selected to represent each of the locations from the broad-scale survey within which *Perkinsus* or suspected-*Perkinsus* infections had been detected, the aim being to confirm *P. olseni* within each of these locations. The 8 *Perkinsus*-negative samples were all from North Jervis Bay and South Jervis Bay, locations where *Perkinsus* was most prevalent. Prior to shipment the samples were preserved for at least 48 h in 10 volumes of uncontaminated, 100% ethanol in sterile, screw-capped containers.

Two PCR assays targeting the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) gene complex, one genus-specific and the other specific to *P. olseni*, were done to detect the presence of *Perkinsus* sp. or *P. olseni* using DNA extracted from the abalone gill and mantle tissues. The gene sequences for *P. olseni* were from samples collected in New Zealand, Australia, Korea, Japan, China and Europe. Primers were designed to sequences that were conserved among the *P. olseni* cultures, but differed from all other sequences. Positive controls were DNA purified from *P. olseni* cells. Negative controls were no target DNA reactions. Amplification products were sequenced to confirm that the sequences matched those of the *P. olseni* sequences.

Screening for *Perkinsus* sp. DNA was performed using *Perkinsus* genus-specific ITS ribosomal RNA complex primers from Casas *et al.* (2002). The forward primer PerkITS-85 (5' CCGCTTTGTTTGGATCCC 3') and reverse primer PerkITS-750 (5' ACATCAGGCCTTCTAATGATG 3') target the ITS region of the rRNA gene complex. Each PCR reaction contained the following: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, each primer at 0.1 µM, 0.025 U µl^{-1} Taq polymerase, 0.05 mg ml^{-1} BSA, and 0.5 µl genomic DNA (10 – 50 ng total). Amplifications were performed with initial denaturation at 95 °C for 4 min followed by 40 cycles of 95 °C for 1 min, 55 °C for 1 min, 65 °C for 3 min, with a final elongation of 65 °C for 5 min. Following amplification, 4 µl of PCR product was visualized on a 2% agarose gel.

DNA from animals shown to have positive amplification products with the *Perkinsus* genus-specific assay was tested in *P. olseni* species-specific PCR assays. *Perkinsus olseni*-specific primers Pols-140F (5' GAC CGC CTT AAC GGG CCG TGT T 3') and PolsITS-600R (5' GGR CTT GCG AGC ATC CAA AG 3') were used in 25-µl reactions. PCR reactions for the *P. olseni* ITS region contained the following: PCR buffer at a concentration of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, each primer at 0.1µM, 0.025 U/µl Taq polymerase, 0.05 mg ml^{-1} BSA and 0.5 ml genomic DNA (~10 – 50 ng). Thermocycling parameters were as follows: an initial denaturation of 95°C for 4 minutes followed by 40 cycles of: 94°C for 1 minute, 62°C for 1 minute, 65°C for 3 minutes, all followed by a final elongation step of 65°C for 10 minutes. Following amplification, for each species-specific reaction, 4 µl of PCR product were analysed as described above.

Genus-specific and *P. olseni*-specific PCR assays, as described above, were also done on tissue samples remaining after the initial tests. Using this second set of tissue samples, PCR assays were done as for the initial tests but, in addition, were done with a 1:10 diluted template. Again, amplification products were sequenced to confirm that sequences matched (or did not match) *P. olseni* sequences.

Control PCR reactions with general SSU primers were used to assess non-target DNA quality. Of the tissue samples from 40 abalone on which PCR assays were attempted, acceptable amplification of SSU DNA was achieved with 31 of the samples. No amplification of SSU DNA was achieved for the other 9 samples. This may have resulted from: (i) poor quality DNA, a consequence of tissue degradation during thawing and refreezing processes or (ii) the presence of inhibitors in the abalone tissue affecting amplifiability of the DNA (K. Reece, pers. comm.). The 31 successful assays were achieved with tissue samples from 7 abalone that tested negative by Ray's test, 6 that were Grade 0.5, 12 that were Grade 1 and 6 that were Grade 2 infections diagnosed using Ray's test. Thus, the range of infection intensities, determined by Ray's test, was still well represented in the sample of 31. Fortunately the 31 successful assays also comprised samples from all 7 locations within which suspected-*Perkinsus* had been detected by Ray's test. Thus, there was still the possibility of confirming *Perkinsus* sp. infections (identified by histology) as *P. olseni* and suspected-*Perkinsus* infections (identified by Ray's test) as *Perkinsus* sp. or *P. olseni* infections at these locations.

4.2.1.4. Data analyses

Chi-squared and Fisher's Exact tests were used extensively to test for differences in frequencies of events in contingency tables. If expected frequencies in cells of contingency tables were < 5, indicating unreliability of a Chi-squared test, then this mandated the use of Fisher's Exact test. It was not possible to use Fisher's Exact test for all contingency tables because of computation limitations (total cell counts > 200 or many cells in the contingency table).

Statistically significant differences in proportions of abalone that tested positive for *Perkinsus* using different diagnostic tests (Ray's, histology, PCR) were identified using McNemar's test (for correlated proportions in the marginals of a 2x2 contingency table) and Chi-square or Fisher's Exact tests where appropriate. Chi-square and Fisher's Exact tests, where appropriate, were used to detect differences between and among: prevalence of suspected-*Perkinsus* infections among locations in 2005; prevalence of infections among sites within locations in 2005; prevalence of infections in 2005 compared to 2002; and intensity of infections in 2005 compared to 2002.

4.2.2. Auxiliary surveys

4.2.2.1. Survey of prevalence and intensity of Perkinsus infections at a smaller spatial scale

Distances between sites sampled during the main survey were typically > 2 km apart. A survey, auxiliary to the main survey, was done over a smaller spatial scale (100's of metres between areas) at Beecroft Head, one of the sites within the location North Jervis Bay, where remnant populations of abalone remained and *Perkinsus* infections were both prevalent and intense. The dual aims of this additional survey were to: (i) determine whether the prevalence and intensity of *Perkinsus* infections in abalone were similar among the Beecroft Head site and areas approximately 250m and 500m both to the north and south of the main site; (ii) provide additional abalone, likely to have relatively intense (high-grade) infections (based on results from the main survey), for histopathology. This survey clearly does not represent a conclusive study of the prevalence of *Perkinsus* infections at the scale of 250 m sections of coast. Sampling areas around replicate sites on the coast would be required to address such an objective. It is, however, a pilot study for any survey that does address the issue of infection prevalence at a similar spatial scale. It is also

relevant that 3 sites surveyed further to the south had few or no abalone thus limiting the extent of our sampling – such a scenario would need to be considered for future studies.

A minimum of 30 abalone with shell lengths between 35 mm and 140 mm were collected from each of the 5 areas (total of 152 abalone) during April 2005. A target sample size of 30 abalone per area would be sufficient to detect one diseased individual (with 95% confidence) if 10% of the population in an area was infected, assuming 100% test sensitivity (i.e., $[1 - 0.10]^{30} = 0.042 < 5\%$). Abalone collected from each area were kept alive, separated and stored within eskies. Methods for data collection, tissue biopsies and diagnostic testing (Ray's test and histology only) were as previously described.

For the purpose of increasing our understanding of the pathogenicity of *Perkinsus* in abalone, histology of abalone collected from this auxiliary survey was only examined if a Ray's test grading of ≥ 1 was detected ($n = 22$). Observations of pathology in this sub-set of abalone was of most interest in studying the pathogenesis of *Perkinsus* in abalone (Chapter 5).

Fisher's Exact tests were used to test for significant differences in proportions of abalone that tested positive to *Perkinsus* by Ray's test and to test for differences in the frequencies of grades of infection intensity among the 5 areas.

4.2.2.2. Survey to assess within-season variation in prevalence of Perkinsus infections

To test whether estimates of the prevalence of suspected-*Perkinsus* from the broad-scale survey may have been dependent on the timing of sampling (i.e., early or late in the summer – autumn season), populations of abalone at 4 sites that were sampled in February were re-sampled during April 2005. A minimum of 20 abalone per site (total of 81 abalone) between 35 and 137 mm SL were collected from the sites North Beecroft and Beecroft Head (within location North Jervis Bay), site South Bowen Island (location South Jervis Bay) and site Sussex Inlet (location Ulladulla). Abalone sampled from the 2 sites at North Jervis Bay were diagnosed with suspected-*Perkinsus* infections during February and no infections were detected in those sampled from South Bowen Island and Sussex inlet during February.

Methods for data collection, tissue biopsies and the use of Ray's test for diagnosis of suspected-*Perkinsus* infections were as previously described (Sections 4.2.1.2 and 4.2.1.3).

Fisher's Exact tests were used to test for significant differences in proportions of abalone that tested positive for suspected-*Perkinsus* by Ray's test and to test for differences in the frequencies of grades of infection intensity between February and April samples.

4.2.3. Grading infection intensity in preserved samples of gill tissue from the 2002 survey

When Ray's test was used to diagnose suspected-*Perkinsus* infections in abalone sampled during 2002, only the presence/absence of suspected-*Perkinsus* cells (hypnospores) was recorded. So that we could compare infection intensities (grades from Ray's test) from the 2005 survey with equivalent data from the 2002 survey, we re-examined archived gill tissue samples (preserved in 95% ethanol) from the 2002 survey.

4.3. Results

4.3.1. Comparative sensitivity of Ray's, histology and PCR tests

A total of 209 abalone tested for suspected-*Perkinsus* using Ray's test were also tested by histology. Eighty-seven of these tested positive by Ray's test and 29 of these also tested positive for *Perkinsus* sp. by histology. There were no instances in which abalone tested positive by histology but negative by Ray's test (Table 4.2 – panel A). The proportion of positive test results by Ray's test (41.6%, 87/209) was significantly greater than the proportion of positive test results by histology (13.9%, 29/209) ($P = 0.000 < 0.01$, McNemar test). Histological examination as a test for the presence of *Perkinsus* sp. detected only 33% of the infections detected by Ray's test.

A total of 31 abalone were tested for *Perkinsus* by both Ray's test and PCR. Twenty-four of these 31 abalone tested positive by Ray's test and 13 of these animals tested positive by PCR. There were no instances in which abalone tested positive by PCR but negative by Ray's test (Table 4.2 – panel B). The proportion of positive test results by Ray's test (77.4%, 24/31) was significantly greater than the proportion of positive test results by PCR (41.9%, 13/31) ($P = 0.001 < 0.05$, McNemar test). PCR as a test for the presence of *Perkinsus* detected only 54% of the infections detected by Ray's test.

A total of 31 abalone were tested for *Perkinsus* sp. by both histology and PCR. Six of these 31 abalone tested positive by histology and 13 tested positive by PCR. Five abalone tested positive by both histology and PCR, 1 abalone tested positive by histology but negative by PCR and 8 abalone tested negative by histology but positive by PCR (Table 4.2 – panel C). The proportion of positive test results by PCR (41.9%, 13/31) was significantly greater than the proportion of positive test results by histology (19.4%, 6/31) ($P = 0.039 < 0.05$, McNemar test). Histology, as a test for the presence of *Perkinsus* detected only 46% of the infections detected by PCR.

If the intensity of infection, based on grades from Ray' test, is taken into account, it is clear that the relative sensitivity of the 3 tests (Ray's, histology and PCR) is similar for high-grade infections (Ray's grade ≥ 2) (Fig. 4.3). Histology detected 24/25 (96%) and PCR detected 5/6 (83%) of high-grade infections. In contrast, low-grade suspected-*Perkinsus* infections (Ray's grade = 0.5 or 1) were rarely detected by histology (5/62) and detected by PCR (8/18) on less than 50% of occasions (Fig. 4.3). The proportion of *Perkinsus* infections detected by histology was significantly greater for high-grade infections than low-grade infections (Fisher's Exact test, $P = 0.000 < 0.01$). The difference in proportions of low-grade and high-grade infections detected by PCR was not statistically significant ($P = 0.166 > 0.05$, Fisher's Exact test).

Table 4.2. Comparative frequencies of detecting *Perkinsus* by Ray's test, histology and PCR.

A. Ray's test v Histology (n = 209)

	Histology +	Histology -	Total
Ray's +	29	58	87
Ray's -	0	122	122
Total	29	180	209

B. Ray's test v PCR (n = 31)

	PCR +	PCR -	Total
Ray's +	13	11	24
Ray's -	0	7	7
Total	13	18	31

C. Histology v PCR (n = 31)

	PCR +	PCR -	Total
Histology +	5	1	6
Histology -	8	17	25
Total	13	18	31

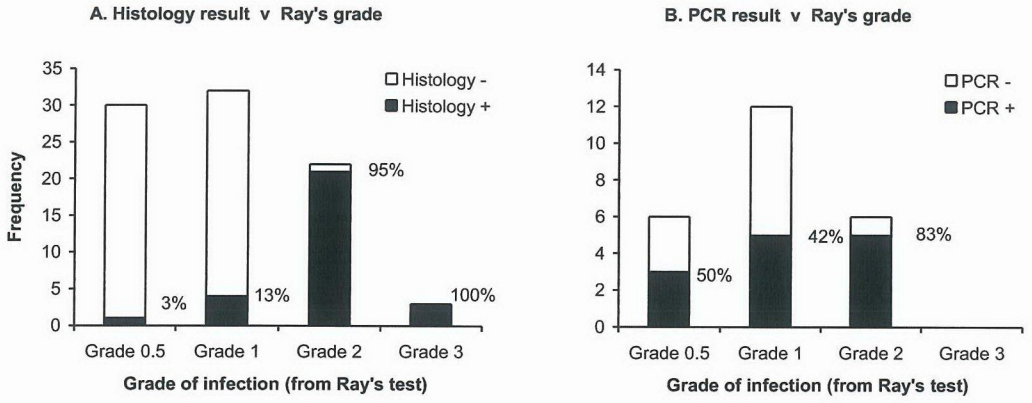

Figure 4.3. Comparative frequencies of detecting *Perkinsus* sp. by histology (panel A) and PCR (panel B) for abalone diagnosed suspected-*Perkinsus* + by Ray's test. Frequencies are shown for 4 grades of infection determined from Ray's test. Percentage figures on graphs represent the % of positive test results.

4.3.2. Geographic range of Perkinsus sp. in 2005 and confirmation of P. olseni

Sixty-five of the 1189 abalone tested using Ray's test were positive for suspected-*Perkinsus*. Thus, suspected-*Perkinsus* was detected at sites within 8 of the 12 locations surveyed (Table 4.3). Seven of the 8 locations were between "North coast" and "South Jervis Bay". All abalone at the location "Forster – Seal Rocks": tested negative for suspected-*Perkinsus* by Ray's test. Positive results by Ray's test were found at 1 of the 4 locations on the south coast between "Ulladulla" and "Tathra-Eden". At "Tathra-Eden" 4 abalone at the Merimbula site tested positive.

Thirteen of 187 abalone tested by histology returned positive results for *Perkinsus* sp. *Perkinsus* sp., identified by histology, were made for abalone collected from all locations between "Central Coast – Sydney" and "South Jervis Bay".

Thirteen of 31 abalone tissue samples successfully tested by genus-specific PCR (i.e., acceptable amplification of SSU DNA) returned positive results for *Perkinsus* sp. In 9 of these 13 positive diagnoses, *P. olseni* was positively identified using the *P. olseni*-specific PCR and/or sequencing of PCR products. In 5 of the 13 positive diagnoses, a *Perkinsus* sp. with a unique ITS gene sequence was identified (and is referred to here as "*Perkinsus* sp.-variant ITS". In 2 instances, results suggested infection of abalone with both *P. olseni* and *Perkinsus* sp.-variant ITS. In a single instance, abalone tissue tested positive by the genus-specific PCR assay, negative by the *P. olseni*-specific PCR and subsequent sequencing was not successful.

The detection of a *Perkinsus* sp. with a previously unknown ITS gene sequence suggests the presence of a new strain of *P. olseni* or a previously undescribed species. The unique ITS region sequences were clearly *Perkinsus,* but in a clade that is sister to the *P. olseni* sequences. These sequences did not group with any of the currently described *Perkinsus* species sequences. Differences within ITS region sequences were approximately 0 – 2.5% within both the *P. olseni* group and the new "unique ITS" group. Between the groups of sequences, variations were approximately in the range 4.5 – 6% (K. Reece, pers. comm.). Due to the uncertainty about the specific taxonomic placement, *Perkinsus* sp. with the unique ITS region sequence, is subsequently referred to as "*Perkinsus* sp.-variant ITS" in this report.

Identification of *P. olseni* and/or *Perkinsus* sp.-variant ITS by PCR was made for 4 of the 5 locations between "Port Stephens" and "South Jervis Bay" (the exception being "North Jervis Bay") and at 2 of the 3 locations to the north of Port Stephens ("Crowdy Head" and "North Coast"). *Perkinsus* sp.-variant ITS was also identified by PCR at the Merimbula site within location "Tathra-Eden", 1 of the 4 locations to the south of "South Jervis Bay" on the south coast of NSW.

Detection of *Perkinsus* infections in abalone within location "Crowdy Head" and as far north as Point Perpendicular (between Crowdy Head and Laurieton, within location "North Coast") represent an extension of the documented range of *Perkinsus* sp. on the north coast of NSW (Table 4.3). Infections of *Perkinsus* were not detected during the 2002 survey at any locations to the north of Port Stephens (Appendix 6 in Section 12.6).

The identification of 4 instances of *Perkinsus* sp. (by Ray's test) and, in one of these instances, identification of *Perkinsus* sp.-variant ITS (by PCR and gene sequencing) at a single site (Merimbula) within the 4 locations between Ulladulla and Tathra-Eden also represents an extension of the range of *Perkinsus* on the south coast of NSW (Table 4.3). *Perkinsus* was not detected during the 2002 survey at any locations to the south of "South Jervis Bay" (Appendix 6 in Section 12.6).

In summary, *P. olseni* and/or *Perkinsus* sp.-variant ITS was confirmed by PCR for abalone within the geographic range "North Coast" – "Crowdy Head", "Port Stephens / Broughton Is." – "South Jervis Bay", but excluding "North Jervis Bay", and at the single site "Merimbula" on the far south coast of NSW. *Perkinsus* sp. was confirmed by histology at "North Jervis Bay". Thus, the 2005 survey detected *P. olseni* and/or *Perkinsus* sp.-variant ITS to the north of the previously documented range of suspected-*Perkinsus* infections, as far north as Point Perpendicular (near Laurieton) and at a single site on the far south coast of NSW, a distance of approximately 200 km south of the previously documented southern limit of distribution of suspected-*Perkinsus* in NSW.

Note that, within the documented geographic range of *Perkinsus* sp., *P. olseni* was identified by PCR between Port Perpendicular (within location "North Coast") and St. Georges Head (within location "South Jervis Bay"). *Perkinsus* sp.-variant ITS was identified by PCR between Crowdy Head North (within location "Crowdy Head") and St. Georges Head (within location "South Jervis Bay") and on the far south coast at Merimbula (within location "Tathra / Eden"). The documented geographic ranges of *P. olseni* and *Perkinsus* sp.-variant ITS represent overlapping components of the documented geographic range of the genus *Perkinsus*.

Moreover, in the 5 instances in which *Perkinsus* sp.-variant ITS was identified, there was no positive identification of *Perkinsus* by histology. This has obvious consequences for determining the pathogenicity of *Perkinsus* sp.-variant ITS . Given the absence of associated histopathology, it cannot be concluded that *Perkinsus* sp.-variant ITS is pathogenic (see Section 5.3.2 in Chapter 5).

The proportion of abalone that tested positive for suspected-*Perkinsus* by Ray's test from the 2005 survey (5.5%, 65/1189, Table 4.3) was not significantly different to the proportion that tested positive from the 2002 survey (5.5% , 44/800, Appendix 6 in Section 12.6). Comparing proportions of abalone infected from the 2002 and 2005 surveys and restricting the comparison to locations that were common to the two surveys, there was no significant difference between 2002 and 2005 (2002: 5.5%, 44/800; 2005: 39/888 from Appendix 6 in Section 12.6; Chi-squared = 1.105, 1 *df*, $P = 0.29$).

4.3.3. Spatial patterns of Perkinsus infection among locations and sites

Proportions of abalone infected with suspected-*Perkinsus* based on Ray's test, differed among locations (Chi-squared = 88.98, $df = 9$, $P < 0.01$) (Fig. 4.4). Data for locations "Crowdy Head" and "Forster – Seal Rocks" were pooled, as were data from "North Bateman's Bay and "South Bateman's Bay" so that a valid Chi-squared test, with expected values in cells of the contingency table > 5, could be done. Proportions of abalone infected with suspected-*Perkinsus* were greatest at locations "North Coast" (22/100, 22%), "North Jervis Bay" (11/102, 11%) and "South Jervis Bay" (13/120, 11%) (Fig. 4.4 and Table 4.3). Proportions of infected abalone at "Crowdy Head" and between "Port Stephens – Broughton Is." and "Kiama" ranged from 2% to 6%. Of the 280 abalone sampled at locations between "Ulladulla" and "South Bateman's Bay" and the 81 sampled at "Forster – Seal Rocks", none tested positive for suspected-*Perkinsus* infections by Ray's test. In 4 of 118 (3%) abalone sampled at the most southern location, "Tathra – Eden", suspected-*Perkinsus* was detected (Fig. 4.4 and Table 4.3).

There were also significant differences in the proportion of abalone infected with suspected-*Perkinsus* among sites within locations (Fig. 4.5). Differences among sites were significant for 4 of the 12 locations: "North Coast", "North Jervis Bay", "South Jervis Bay" and "Tathra – Eden" (Fisher's Exact tests, $P < 0.01$). At location "North Coast", 75% of abalone (15/20) at one site (Diamond Head) were infected with suspected-*Perkinsus*. Between 15% and 20% of abalone were infected at 2 other sites within this location and there were no infections detected at the 2 remaining sites. At both "North Jervis Bay" and "South Jervis Bay", suspected-*Perkinsus* was detected at 3 sites with one site at each location within which greater than a third of abalone were

infected. At the southern most survey location, "Tathra – Eden", abalone infected with suspected-*Perkinsus* were detected at a single site ("Merimbula") at which 20% (4/20) were infected.

It is noteworthy that within location "South Jervis Bay", 12 of the 13 abalone identified with suspected-*Perkinsus* infections came from the 2 most southern sites ("St George's Head" and "St Georges Head South"). In contrast, all 19 infections identified within this location in 2002 came from the 4 sites to the north of "St George's Head" (Appendix 6 in Section 12.6). Location "South Jervis Bay" was identified as the southern front of suspected-*Perkinsus* infections in NSW 2002. Location "Port Stephens – Broughton Is." was identified as the northern "front" of infections in the 2002 survey within which, the northernmost site at which suspected-*Perkinsus* was detected was "South Fingal Is". In 2002, no infections of suspected-*Perkinsus* (0/40 abalone) were found at the 2 sites to the north of "South Fingal Is" ("Shag Rock, Broughton Is." and "Elizabeth Bay, Broughton Is.") (Appendix 6 in Section 12.6). In contrast, 4/20 abalone sampled from "Elizabeth Bay, Broughton Is." in 2005 were infected. Within both the locations identified as northern and southern "fronts" of infection in 2002, infections were detected in 2005 at sites further to the north and south, respectively.

Table 4.3. Prevalence of *Perkinsus* infections detected at sites and locations during the 2005 broad-scale survey, using Ray's test (for suspected-*Perkinsus*), histology (for *Perkinsus* sp.) and PCR/gene sequencing (for *Perkinsus* sp., *P. olseni* or *Perkinsus* sp.-variant ITS). Note that histology and PCR tests were done on sub-samples of the abalone for which Ray's test was done. The sample size shown for PCR in the format "a (b)" indicates that PCR was attempted on "b" abalone but that acceptable amplification of non-target SSU DNA and therefore valid test results were only achieved for "a" abalone. The column titled "Conclusion re *Perkinsus* at location" indicates whether (i) *P. olseni* and/or *Perkinsus* sp.-variant ITS have been identified by PCR and gene sequencing and/or (ii) *Perkinsus* sp. has been identified by histology/PCR within a location.

Location	Site	Month	N	Ray's test						Histology		PCR & gene seq.				Conclusion re *Perkinsus* at Location
				n	Grade				+	n	+	n	*P.* sp.	*P. olseni*	*P.* sp variant ITS	
					0.5	1	2	3								
Nth Coast	Bonnie Hills	5	20	20	0	0	0	0	0	2	0	0				
	Grants Head	5	20	20	0	0	0	0	0	1	0	0				
	Point Perpendicular	5	20	20	3	1	0	0	4	8	0	2 (2)	1	1	0	
	Diamond Head Bay	5	20	20	3	0	0	0	3	19	0	1 (2)	1	0	0	
	Diamond Head	5	20	20	9	6	0	0	15	20	0	3 (4)	0	0	0	
	TOTAL		**100**	**100**	**15**	**7**	**0**	**0**	**22**	**50**	**0**	**6 (8)**	**2**	**1**	**0**	***Perkinsus* sp.** ***P. olseni***
Crowdy Head	Crowdy Nth	3	21	21	0	2	0	0	2	4	0	1 (2)	1	0	1	
	Crowdy Sth	3	20	20	0	2	0	0	2	4	0	1 (2)	0	0	0	
	Wallabi Point	3	20	20	0	0	0	0	0	1	0	0				
	Red Head	3	20	20	0	0	0	0	0	1	0	0				
	TOTAL		**81**	**81**	**0**	**4**	**0**	**0**	**4**	**10**	**0**	**2 (4)**	**1**	**0**	**1**	***Perkinsus* sp.** ***Perkinsus* sp.-variant ITS**
Forster/ Seal Rx	Forster (Lattitude Rock)	3	21	21	0	0	0	0	0	1	0	0				
	Seal Rocks (Lighthouse)	3	21	20	0	0	0	0	0	2	0	0				
	Seal Rocks (Treachery)	3	20	20	0	0	0	0	0	1	0	0				
	Yagan	3	20	20	0	0	0	0	0	1	0	0				
	TOTAL		**82**	**81**	**0**	**0**	**0**	**0**	**0**	**5**	**0**	**0**				negative

Table 4.3. (Cont'd)

Location	Site	Month	N	Ray's test						Histology		PCR & Seq.				Conclusion re *Perkinsus* at Location
				n	Grade				+	n	+	n	*P.* sp.	*P. olseni*	*P.* sp variant ITS	
					0.5	1	2	3								
Port Stephens/ Broughton Is	Elizabeth Bay (Broughton Is)	2	20	20	3	1	0	0	4	8	0	2 (4)	1	1	1	
	Tomaree	2	20	20	0	0	0	0	0	1	0	0				
	South Fingal Is	2	20	20	1	0	0	0	1	2	0	0				
	Boulder Bay	2	20	20	0	0	0	0	0	1	0	0				
	Boat Harbour	2	20	20	1	0	0	0	1	2	0	0				
	TOTAL		**100**	**100**	**5**	**1**	**0**	**0**	**6**	**14**	**0**	**2 (4)**	**1**	**1**	**1**	***Perkinsus* sp.** ***P. olseni*** ***Perkinsus* sp.-variant ITS**
Central Coast/ Sydney	Forresters	1	22	22	0	0	1	0	1	2	1	0				
	Terrigal (Skillion)	1	21	21	0	0	0	0	0	1	0	0				
	Newport	2	20	20	0	0	2	0	2	4	1	2 (2)	2	2	0	
	Sand Shoes	1	21	21	0	0	0	0	0	1	0	0				
	Curracarang	1	19	19	0	0	0	0	0	1	0	0				
	TOTAL		**103**	**103**	**0**	**0**	**3**	**0**	**3**	**9**	**2**	**2 (2)**	**2**	**2**	**0**	***Perkinsus* sp.** ***P. olseni***
Kiama	Bass Point	1	20	20	0	0	0	0	0	1	0	0				
	The Farm	1	20	20	0	0	0	0	0	1	0	0				
	Little Blowhole	1	20	20	0	0	0	0	0	1	0	0				
	Obelisk	1	20	20	0	0	1	0	1	2	1	1 (1)	1	1	0	
	Gerroa	1	20	20	1	0	0	0	1	2	0	0				
	TOTAL		**100**	**100**	**1**	**0**	**1**	**0**	**2**	**7**	**1**	**1 (1)**	**1**	**1**	**0**	***Perkinsus* sp.** ***P. olseni***
North Jervis Bay	Nth Beecroft[R]	2	21	21	0	0	1	0	1	2	1	0				
	Beecroft Head[R]	2	20	20	2	3	2	0	7	14	2	5 (6)	0	0	0	
	Targets	2	20	20	2	0	0	1	3	6	1	0 (1)	0	0	0	
	Drum & Drum Sticks	2	20	20	0	0	0	0	0	1	0	0				
	Croc Head	2	21	21	0	0	0	0	0	1	0	0				
	TOTAL		**102**	**102**	**4**	**3**	**3**	**1**	**11**	**24**	**4**	**5 (7)**	**0**	**0**	**0**	***Perkinsus* sp.**
South Jervis Bay	Sth Bowen Is[R]	2	20	20	0	0	0	0	0	2	0	0				
	Nth Stoney Creek	2	20	20	0	0	0	0	0	1	0	0				
	Sth Stoney Creek	2	20	20	0	1	0	0	1	2	0	0				
	Sth Steamers	2	20	20	0	0	0	0	0	1	0	0				
	St Georges Head	2	20	20	2	5	4	0	11	20	6	10 (10)	5	4	2	
	St Georges Sth	4	20	20	0	1	0	0	1	2	0	0				
	TOTAL		**120**	**120**	**2**	**7**	**4**	**0**	**13**	**28**	**6**	**10 (10)**	**5**	**4**	**2**	***Perkinsus* sp.** ***P. olseni*** ***Perkinsus* sp.-variant ITS**

Table 4.3. (Cont'd)

Location	Site	Month	N	Ray's test						Histology		PCR & Seq.				Conclusion re *Perkinsus* at Location
				n	Grade				+	n	+	n	*P.* sp.	*P. olseni*	*P.* sp variant ITS	
					0.5	1	2	3								
Ulladulla	Sussex Inlet[R]	1	20	20	0	0	0	0	0	1	0	0				
	Bendalong	1	20	20	0	0	0	0	0	1	0	0				
	Green Island	1	20	20	0	0	0	0	0	1	0	0				
	Bannister Head	1	20	20	0	0	0	0	0	1	0	0				
	Ulladulla (Lighthouse)	1	20	20	0	0	0	0	0	1	0	0				
	TOTAL		**100**	**100**	**0**	**0**	**0**	**0**	**0**	**5**	**0**	**0**		**0**		negative
Nth Bateman's Bay	Nuggan Point	3	20	20	0	0	0	0	0	1	0	0				
	Brush Island	3	20	20	0	0	0	0	0	1	0	0				
	Kioloa (Belowla Is)	3	20	20	0	0	0	0	0	1	0	0				
	Snapper	3	20	20	0	0	0	0	0	2	0	0				
	Pretty (O'Hara Is)	3	20	20	0	0	0	0	0	1	0	0				
	TOTAL		**100**	**100**	**0**	**0**	**0**	**0**	**0**	**6**	**0**	**0**		**0**		negative
Sth Bateman's Bay	Mossy Point	4	20	20	0	0	0	0	0	1	0	0				
	Narooma Break	4	20	20	0	0	0	0	0	1	0	0				
	Mystery Bay	4	20	20	0	0	0	0	0	1	0	0				
	Bermagui	4	20	20	0	0	0	0	0	1	0	0				
	TOTAL		**80**	**80**	**0**	**0**	**0**	**0**	**0**	**4**	**0**	**0**		**0**		negative
Tathra/ Eden	Tathra (Bunga)	5	21	21	0	0	0	0	0	1	0	0				
	Tathra (Mini pressure)	5	20	20	0	0	0	0	0	1	0	0				
	Merimbula	4	20	20	3	1	0	0	4	20	0	3 (4)	1	0	1	
	Eden	4	22	22	0	0	0	0	0	1	0	0				
	Disaster Bay	4	19	19	0	0	0	0	0	1	0	0				
	Wonboyn	4	20	20	0	0	0	0	0	1	0	0				
	TOTAL		**122**	**122**	**3**	**1**	**0**	**0**	**4**	**25**	**0**	**3 (4)**	**1**	**0**	**1**	***Perkinsus* sp.** ***Perkinsus* sp.-variant ITS**
	GRAND TOTAL		**1190**	**1189**	**30**	**23**	**11**	**1**	**65**	**187**	**13**	**31 (40)**	**13**	**9**	**5**	

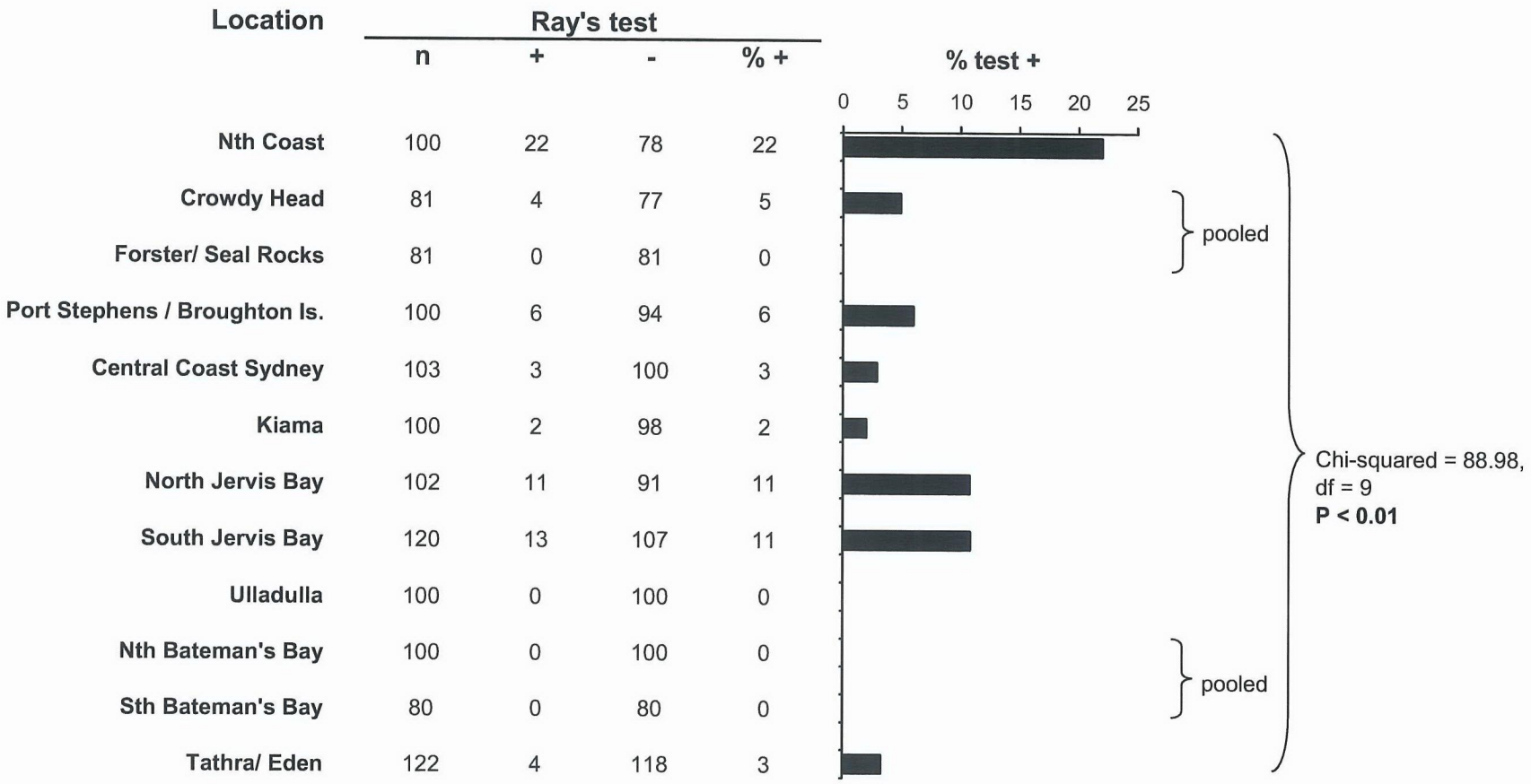

Figure 4.4. Variation in prevalence of suspected-*Perkinsus* infections (positive Ray's test results) among locations from the 2005 broad-scale survey.

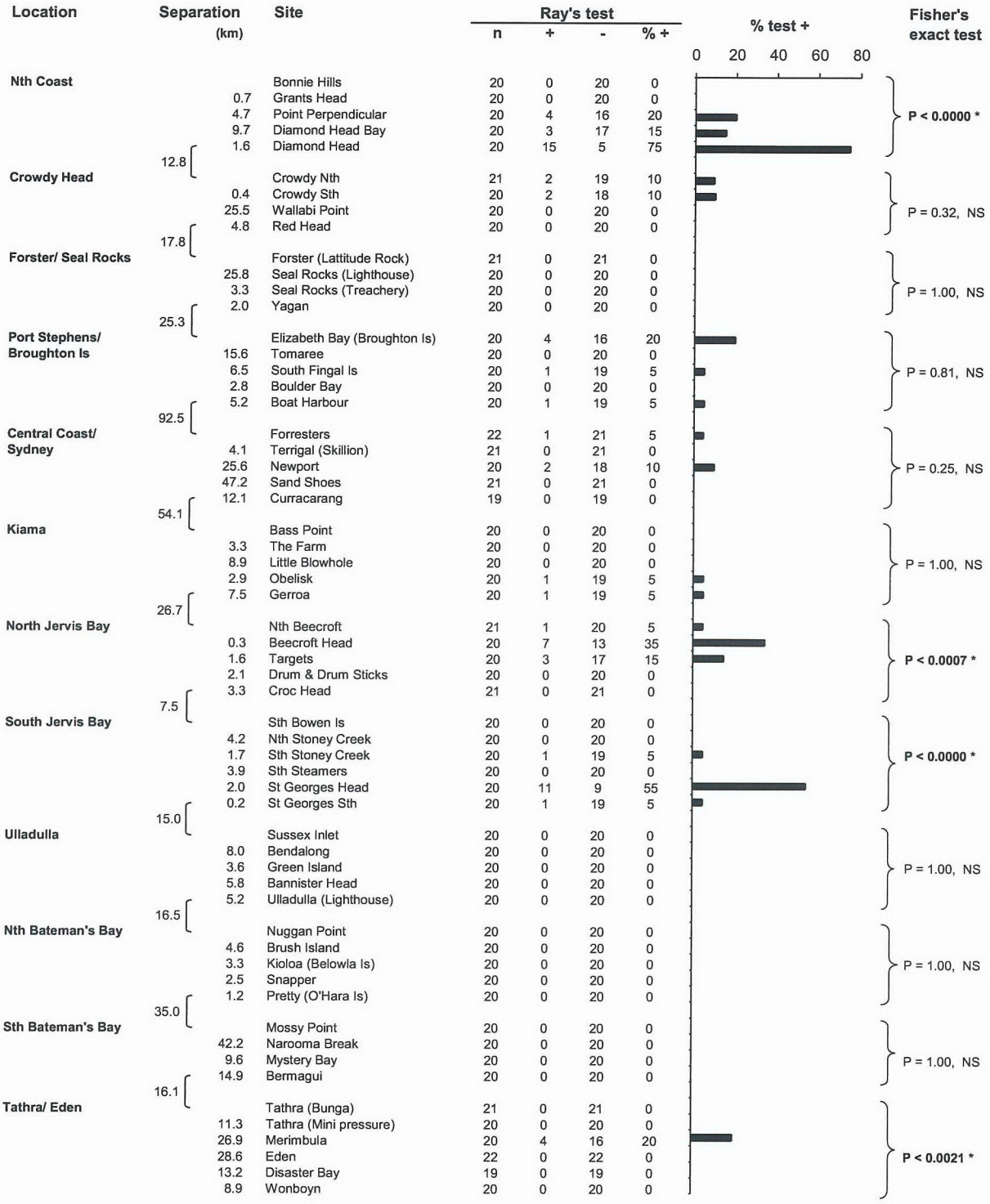

Figure 4.5. Variation in prevalence of suspected-*Perkinsus* infections (positive Ray's test results) among sites within locations from the 2005 broad-scale survey. The listed probability from the Fisher's Exact test among sites at each location is for the test applied at that single location. To control type-I error across the 12 tests (i.e., 12 locations), using the "Bonferroni method", probabilities for individual tests must multiplied by the number of tests done. All statistically significant test results indicated here (*) still apply following this procedure.

4.3.4. Patterns of Perkinsus infection at a smaller spatial scale – pilot survey at Beecroft Head

During the broad-scale survey in 2005, 35% (7/20) of abalone sampled from the site "Beecroft Head" (within the location "North Jervis Bay") were infected with suspected-*Perkinsus*. When re-sampled in April 2005, 26% (8/31) of abalone were infected (not significantly different to the original sample, Fisher's Exact test, $P = 0.54$).

There was no significant difference in the proportions of abalone infected with suspected-*Perkinsus* at "Beecroft Head" and areas of reef 250m to the north, 500m to the north, 250m to the south and 500m to the south of the "Beecroft Head" site (Fisher's Exact test, $P = 0.367$). Neither was there significant difference in the proportions of the various grades of infection intensity among these areas (Fisher's Exact test, $P = 0.081$). Thus, populations of abalone on these 5 areas of reef within 1 km stretch of coastline had a similar incidence of suspected-*Perkinsus* infections and distribution of intensities of infection in affected abalone (Table 4.4).

Table 4.4. Prevalence and intensity of suspected-*Perkinsus* infections (determined using Ray's test) in abalone from Beecroft Head and 4 areas 250m and 500m to both the north and south.

Site	Area (relative to Site)	Ray' test						
		n	+	-	Grade			
					0.5	1	2	3
Beecroft Head	500m north	30	5	25	2	1	2	0
	250m north	30	9	21	5	1	3	0
	Beecroft Head	31	8	23	3	0	4	1
	250m south	30	9	21	1	6	2	0
	500m south	31	4	27	2	1	0	1
		152	35	117	13	9	11	2

4.3.5. Intensity of Perkinsus infections

In the broad-scale survey, 65 of the 1189 abalone tested using Ray's test were identified with suspected-*Perkinsus* infections. Intensity of infection, indicated by the grade classifications from Ray's test, varied among the 65 abalone. There was a significant difference among the proportions of infected abalone classified in the various grades of infection from Ray's tests (Chi-square = 40.59, $df = 3$, $P < 0.01$). Of the 65 abalone that tested positive using Ray's test: 46% (30/65) were classified as Grade 0.5; 35% (23/65) as Grade 1; 17% (11/65) as Grade 2; and 2% (1/65) as Grade 3 infections (Fig. 4.5). Collapsing these grades into "high-grade" (grade ≥ 2) and "low-grade" (grades 0.5 and 1) categories, 82% (53/65) of infected abalone had low-grade and 18% (12/65) had high-grade infections.

Intensities of infection in abalone also differed among locations. Of the abalone that tested positive by Ray's test, proportions classified as low-grade and high-grade infections differed significantly among locations (Fisher's Exact test, $P < 0.01$). High-grade (Ray's grade ≥ 2) infections were only detected between "Central Coast – Sydney" and "South Jervis Bay" (Fig. 4.6). Within these locations, 59% (17/29) infected abalone had low-grade infections and 41% (12/29) were classified

as high-grade. Infections detected to the north of these locations and at the single site within "Tathra – Eden" to the south were low-grade (Fig. 4.6).

In the 5 instances in which *Perkinsus* sp.-variant ITS was identified by PCR and gene sequencing, the intensities of infection were low-grade (Ray's Grade 0.5 or 1). In contrast, in the 9 instances in which *P. olseni* by PCR and gene sequencing, 5 high-grade (all Ray's Grade 2) and 4 low-grade (Ray's grade 0.5 or 1) infections were determined by Ray's test.

Although there was no significant difference in the proportion of abalone infected with suspected-*Perkinsus* in 2005 compared to 2002 (Section 4.3.2), there was a significant difference in the intensity of infections in 2005 compared to 2002. Grades of infection ranged between 0.5 and 3 in 2005 but between 1 and 5 in 2002 (Fig. 4.7). Restricting the analysis to locations common to both surveys, proportions of positive Ray's test results assigned different grades differed significantly between years (Fisher's Exact test, $P < 0.01$). In 2005, only 31% (12/39) of infections were classified as high-grade whereas 95% (39/41) of infections in 2002 were high-grade. No Grade 4 or Grade 5 infections were detected by Ray's test in 2005, but 26% (10/39) were detected in 2002 (Fig. 4.7, Appendix 6 in Section 12.6).

The differences in intensity of infections between 2002 and 2005 explain the better overall relative sensitivities of Ray's test and histology in 2002 compared to 2005 (see Section 4.3.1). The relative sensitivity in 2005 of histology compared to Ray's test for detecting *Perkinsus* was good (96%) for high-grade infections but poor for low-grade infections (8%). The proportion of low-grade infections in 2005 was much greater than in 2002. For all grades of infection pooled, the relative sensitivity of histopathology in 2005 was only 33% (29/87) while in 2002 histopathology detected more cases than Ray's test (16 v 13). If, however, the relative sensitivity of these 2 tests compared between 2002 and 2005 is restricted to high-grade infections, then relative sensitivity of histology compared to Ray's test was not significantly different between years (2002: 90%, 9 Histo + / 10 Ray's +; 2005: 96%, 24 Histo + / 25 Ray's +; Fisher's Exact test, $P = 0.50$).

4.3.6. Within-season variation in prevalence of Perkinsus infections

No suspected-*Perkinsus* infections were detected in abalone at 2 of the 4 sites ("South Bowen Is" and "Sussex Inlet") surveyed during both February and April 2005. At the other 2 sites ("North Beecroft" and "Beecroft Head", within location "North Jervis Bay"), there were no significant differences between proportions of abalone infected with suspected-*Perkinsus* during February and April (Fisher's Exact tests, $P > 0.05$ for February v April for individual sites and both sites pooled) (Table 4.5). Neither were there significant differences in the grades of infection between February and April (Fisher's Exact tests, $P > 0.05$ for February v April for individual sites and both sites pooled) (Table 4.5). Note, however, the non-significant (Fisher's Exact test, $P = 0.152$) trend for infections to be of greater intensity during April compared to February at "Beecroft Head" (Table 4.5).

Location	Num. Abalone	Num. Ray's +	Grade of Ray's test result 0.5	1	2	3
North Coast	100	22	15	7	0	0
Crowdy Head	81	4	0	4	0	0
Forster / Seal Rocks	81	0	0	0	0	0
Port Stephens / Broughton Is	100	6	5	1	0	0
Central Coast / Sydney	103	3	0	0	3	0
Kiama	100	2	1	0	1	0
North Jervis Bay	102	11	4	3	3	1
South Jervis Bay	120	13	2	7	4	0
Ulladulla	100	0	0	0	0	0
North Bateman's Bay	100	0	0	0	0	0
South Bateman's Bay	80	0	0	0	0	0
Tathra / Eden	122	4	3	1	0	0
	1189	**65**	**30**	**23**	**11**	**1**

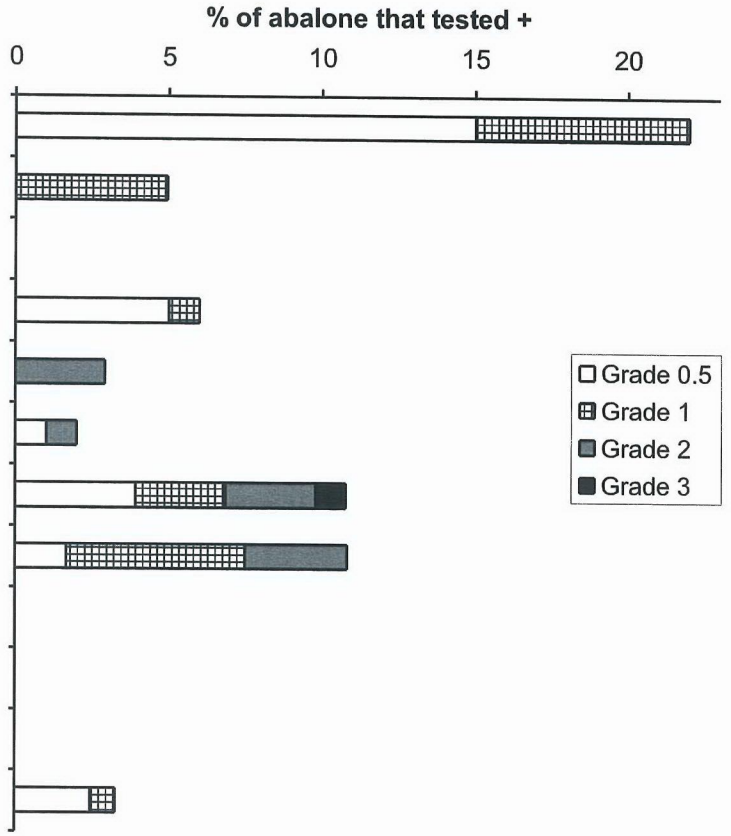

Figure 4.6. Variation in the intensity of suspected-*Perkinsus* infections (grades from Ray's test) among locations sampled during the 2005 broad-scale survey.

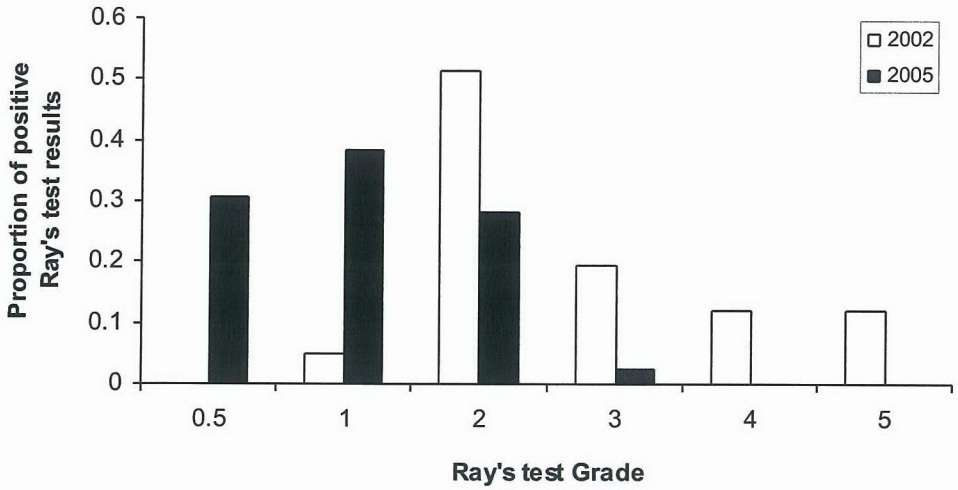

Figure 4.7. Comparison of the intensity of suspected-*Perkinsus* infections (grades from Ray's test) from the 2002 and 2005 broad-scale surveys.

Table 4.5. Comparison of prevalence and intensity of suspected-*Perkinsus* infections (determined using Ray's test) at 4 sites surveyed in February and in April 2005.

Location	Site	February 2005							April 2005						
		Rays test							Rays test						
		N	+	-	Grade				N	+	-	Grade			
					0.5	1	2	3				0.5	1	2	3
North Jervis Bay	North Beecroft	21	1	20	0	0	1	0	20	3	17	1	1	1	0
	Beecroft Head	20	7	13	2	3	2	0	21	4	17	0	0	3	1
		41	8	33	2	3	3	0	41	7	34	1	1	4	1
South Jervis Bay	South Bowen Is	20	0	20	0	0	0	0	20	0	20	0	0	0	0
Ulladulla	Sussex Inlet	20	0	20	0	0	0	0	20	0	20	0	0	0	0

4.4. Discussion

4.4.1. Performance of diagnostic methods

Compared to histology (on multiple organs/tissues) and PCR (on gill and mantle tissues), Ray's test on samples of gill tissue from abalone sampled in 2005, was the most sensitive for detecting *Perkinsus* infections. Handlinger *et al.* (2006) discussed the similar sensitivity of Ray's test and histology for detecting *Perkinsus* in samples from 2002 but noted the need for further information to confirm the conclusion. In contrast to the 2002 survey, most infections in abalone in 2005 were low-grade and histology generally did not detect infections if the intensity based on Ray's test was $\leq$ Grade 1. Our conclusion that Ray's test is more sensitive than histology for detecting low intensity infections is consistent with conclusions made for infections of *Perkinsus* spp. in other host species (e.g., Almeida *et al.*, 1999; Villalba *et al.*, 2005). Development and study of PCR assays for detecting infections of *P. marinus* has been more extensive than for other species of *Perkinsus*, including *P. olseni* (Villalba *et al.*, 2004). Some studies have demonstrated greater sensitivity of PCR than Ray's test on tissue for detecting *P. marinus* in oysters (*Crassostrea virginica*) in the USA (e.g., Robledo *et al.*, 1998; Yarnall *et al.*, 2000). The lesser sensitivity of histology and the PCR assay compared to Ray's test for detecting low-grade *Perkinsus* infections in the abalone sampled from NSW in 2005 is most likely a consequence of the relative volumes of tissue examined by each method. The quantity of tissue examined in Ray's test was at least 4 times greater than the quantities of tissue examined using histological and PCR methods. In particular, the quantity of gill tissue examined was least for histology, which also examined cross-sections of a range of other tissues. The intensity of *P. marinus* infections has been found to vary within and among tissue types of oysters (Choi *et al.*, 1989). Thus, the relative sensitivity of the 3 different methods, as used in our study for detecting *Perkinsus,* is likely to be a function of the relative diagnostic sensitivities of the 3 assays *per se*, the tissue types and the volume of tissue examined by each of 3 assays.

An important limitation of Ray's test is that it is non-specific and therefore, there is the possibility that we have detected other organisms such as dinoflagellates in addition to *Perkinsus* (e.g., Almeida *et al.*, 1999) and this would affect our comparisons of sensitivity. However, *Perkinsus* sp. was identified by histology and/or PCR in abalone from all locations where Ray's test positives were also detected so this scenario is considered unlikely.

Given the potential advantages of the speed, efficiency and specificity of PCR over Ray's test and histology for detecting *Perkinsus* infections, further development of PCR assays for diagnosis of *P. olseni* and *Perkinsus* sp.-variant ITS infections in abalone in NSW and Australia is indicated. In particular, real-time PCR can be used to more accurately quantify initial parasite levels in tissue than the standard PCR, which relies on an end-point reaction (Gauthier *et al.*, 2006). Some pilot development work of a Taqman real-time PCR for detecting *P. olseni* in molluscs is currently being done at the CSIRO Aquatic Animal Health Laboratories in Geelong and initial results are promising (S. Corbeil, pers. comm.). The need for further development and a formal study of the comparative sensitivity of real-time PCR, histology and Ray's test is discussed in Section 7.3.

The identification, during this project, of a *Perkinsus* sp. with a unique ITS region sequence (referred to as *Perkinsus* sp.-variant ITS here) raises questions about the taxonomy of this *Perkinsus* sp. and also the specificity of the *P. olseni*-specific PCR assay used in this study. If *Perkinsus* sp.-variant ITS represents a previously undescribed strain of *P. olseni*, then the species-specific *P. olseni* PCR assay used here is strain-specific as it did not detect *Perkinsus* sp.-variant ITS. Note that infections of *Perkinsus* sp.-variant ITS were identified by the combination of a positive genus-specific PCR, negative *P. olseni*-specific PCR and subsequent sequencing of amplification products from the genus-specific PCR assay. Alternatively, if *Perkinsus* sp.-variant

ITS represents a previously undescribed species of *Perkinsus*, then development of a PCR specific to this species is indicated.

4.4.2. *Identification of Perkinsus sp., P. olseni and Perkinsus sp.-variant ITS and the geographic range of infections*

Based on the PCR assays and subsequent sequencing of the ITS region, *P. olseni* and/or *Perkinsus* sp.-variant ITS were for the first time, positively identified in abalone from NSW, infecting abalone within 7 of the 8 locations at which suspected-*Perkinsus* was identified by Ray's test. However, the geographic distribution of each strain is not known with certainty due to: (i) the small numbers of abalone that were tested for *Perkinsus* by PCR and (ii) the identification of *P. olseni* at 5 of the 8 locations at which *Perkinsus* infections were detected and the identification of *Perkinsus* sp.-variant ITS at 4 of the 8 locations at which suspected-*Perkinsus* infections were detected. Similarly, if *Perkinsus* sp.-variant ITS is a different species, then the actual geographic distribution of each species is not known with certainty for the same reasons.

Future sampling of abalone from the locations and sites surveyed during this study with PCR assays and gene sequencing on a greater sample size would provide a better understanding of the geographic distribution of *P. olseni* and *Perkinsus* sp.-variant ITS and this is a future research priority (see Section 7.3).

Although there was no confirmation by PCR of *P. olseni* or *Perkinsus* sp.-variant ITS at North Jervis Bay, it seems reasonable to conclude that the identification of the species of *Perkinsus* here is indeed *P. olseni* or *Perkinsus* sp.-variant ITS, given confirmation of *Perkinsus* sp. within this location by histology and given the confirmation of *P. olseni* at Kiama (to the north) and both *P. olseni* and *Perkinsus* sp.-variant ITS at South Jervis Bay (to the south) of North Jervis Bay.

It is therefore concluded that *P. olseni* is one species of the genus *Perkinsus* infecting abalone along the coast of NSW. Evidence from the PCR assays and gene sequencing also suggests the presence of a previously undescribed *Perkinsus* species or variant strain of *P. olseni.* Note, however, that further studies are required regarding the status of *Perkinsus* sp.-variant ITS as a strain of *P. olseni* versus a previously undescribed species of *Perkinsus*. In subsequent sections of this report, when necessary and possible, we distinguish between *P. olseni* and *Perkinsus* sp.-variant ITS. In all other instances, we simply refer to "*Perkinsus*" or "*Perkinsus* sp.". Because *P. olseni* or *Perkinsus* sp.-variant ITS were positively identified in abalone across the geographic range of suspected-*Perkinsus* infections, we dispense with the term "suspected-*Perkinsus*" (positive Ray's test result) and assume that all cases of "suspected-*Perkinsus*" are *Perkinsus* sp.

The geographic range of *Perkinsus* sp. infections in blacklip abalone in NSW that was identified from the broad-scale survey in 2005 was greater than previously documented. Infections were detected in abalone to the north of Port Stephens at Crowdy Head (within location Crowdy Head) and at Diamond Head, Diamond Head Bay and Point Perpendicular (within location "North Coast"). However, no infections were detected within location "Forster – Seal Rocks", the location between "Port Stephens" and "Crowdy Head". On the far south coast, infections were also detected in 4 abalone at the Merimbula site within the southern-most survey location "Tathra-Eden". There were no infections detected at sites within locations "Ulladulla", "North Bateman's Bay" and "South Bateman's Bay", the 3 locations between "Tathra – Eden" and the southern-most detection of *Perkinsus* sp. at "South Jervis Bay" in 2002. Thus, the detection of *Perkinsus* sp. at Merimbula in 2005 represents an "outpost" of infection at a single site amongst the total of 20 sites surveyed to the south of South Jervis Bay in 2005.

Detection of *Perkinsus* sp. infections to the north of Port Stephens and at the Merimbula site on the far south coast, outside of the previously documented range may indicate actual extension of the geographic range of *Perkinsus* sp. in NSW. Alternatively, they may be an artefact of the greater

geographic range of the survey in 2005. The northern extent of the 2002 survey was Crowdy Head and 0/120 abalone tested positive by Ray's test at sites sampled between Port Stephens and Crowdy Head. At comparable locations from the 2005 survey (locations "Forster – Seal Rocks" and "Crowdy Head"), infections were detected in only 4/163 abalone sampled. This is not a statistically significant difference in prevalence. The sites to the north of Port Stephens at which *Perkinsus* sp. was most prevalent in 2005 were all to the north of Crowdy Head within location "North Coast" and there was no sampling on this stretch of coast in 2002. Similarly, at locations on the south coast common to both the 2002 and 2005 surveys, no *Perkinsus* sp. infections were identified at any of the sites within locations "Ulladulla" or "North Bateman's Bay" to the south of Jervis Bay. The identification of *Perkinsus* sp. at the Merimbula site in 2005 was within a location to the south of the southern-most location surveyed in 2002. Whilst this section of coast was surveyed in 2003 (NSW component of the NSDA) to provides samples for histopathology, Ray's test was not done on these samples. Based on our findings of the relative sensitivity of Ray's test and histology, it is unlikely that any low intensity infections of *Perkinsus* sp. in these samples would have been identified.

Thus, given that the 2002 survey did not extend as far north or south as the 2005 survey, we can conclude an extension in the documented geographic range of *Perkinsus* sp. infections in blacklip abalone on the coast of NSW. We cannot conclude an actual extension of the range. Moreover, given that *Perkinsus* sp. was detected in abalone in the northern-most location surveyed in 2005 (location "North Coast", Diamond Head to Bonnie Hills), the presence of the parasite in abalone further north cannot be discounted. Although populations of blacklip abalone are less abundant on the far north coast of NSW, future broad-scale surveys of the prevalence of *Perkinsus* sp. infections in abalone should be extended to include the far north coast of NSW (see Section 7.3).

4.4.3. Spatial and temporal variations in prevalence and intensity of infections

In 2002, prevalence of *Perkinsus* sp. infections tended to be greatest in populations of abalone at the margins of the infected zone, that is, at Port Stephens and at Jervis Bay. Excluding the "outpost" of *Perkinsus* sp. infection at Merimbula, the same pattern is apparent in the 2005 data. Prevalence was greatest within location "North Coast" (22%, 22/100 abalone infected) the northern-most location at which *Perkinsus* sp. was detected. On the south coast, at Jervis Bay, infections were detected in 11% of the population (11/102 and 13/120 at locations "North Jervis Bay" and "South Jervis Bay" respectively). At both the northern "front" and southern (Jervis Bay) "front", there were significant differences in prevalence of *Perkinsus* sp. infections among sites within locations. Within location "North Coast", infections were most prevalent at Diamond Head (75% of abalone sampled were infected) and *Perkinsus* sp. was not detected at the two north-most sites within the location. Within location "South Jervis Bay", a different pattern of prevalence from that documented for 2002 emerged. In 2002, *Perkinsus* sp. was detected in abalone at the 4 most northern sites but not at St. Georges Head. However, in 2005, abalone were scarce at these 4 northern-most sites and prevalence of infections was also less than in 2002 at these sites. Importantly, in 2005 11/20 abalone from the south-most site, St. Georges Head, were infected with *Perkinsus* sp. At locations between these northern and southern "fronts", *Perkinsus* sp. infections were less prevalent (up to 6% within any single location) and there were no significant differences among sites within locations. Note that our sampling strategy provided the most confidence of detecting of *Perkinsus* sp. at the level of location (95% probability of detecting at least one infected abalone if 3% of the population was infected) and lesser confidence at the level of site (46% probability of detecting at least 1 infected abalone if 3% of the population at that site was infected).

The observation of greatest infection prevalence at the northern and southern fronts of infection was apparent from the spatial distribution of infections from the 2002 survey. Callinan and Landos, (2006) noted the significance of this pattern as being epidemiologically consistent with a propagating epizootic entering naïve populations. Persistence of this pattern in 2005 (excluding the

"outpost" of *Perkinsus* sp. infection at Merimbula) with greatest prevalence of infections at the most northern survey location and at the most southern site at South Jervis Bay provides additional support for such a hypothesis (see Chapter 6).

In preparation for considering the epizootiology of perkinsosis (in Chapter 6), it is important to note here, the co-incidence of prevalence and intensity of *Perkinsus* sp. infections in the Jervis Bay region (North and South Jervis Bay). Infections were both prevalent and intense in the vicinity of Jervis Bay in both 2002 and 2005. In contrast, the extremely high prevalence of infections detected at location "North Coast" in 2005 was not associated with high-grade infection intensity. All infections detected at locations including and to the north of Port Stephens were low-grade.

Comparisons of prevalence and intensities of *Perkinsus* sp. infections among years (2002 v 2005) and among sites and locations in 2005 are potentially biased if prevalence and intensity were related to the month during which sampling was done. The broad-scale survey in 2002 was completed during February – May with most sampling during April – May. The broad-scale survey in 2005 ("Crowdy Head" to "North Bateman's Bay") was done from January – April, with most sampling during February – March. Thus, on average, sites were surveyed approximately 2 months earlier in 2005 compared to 2002. Studies of disease progression for *Perkinsus* spp. infecting various hosts in other locations demonstrate associations between water temperature and both prevalence and intensity of infections (e.g., Ragone Calvo *et al.*, 2003b). If, on average, due to changes in water temperature or some other seasonal factor, prevalence and/or intensity of *Perkinsus* sp. infections in abalone differ between February – March and April – May, then comparisons among samples that were collected in different months may be biased. The auxiliary experiment done at 3 sites at Jervis Bay and a single site within an adjacent location, "Ulladulla", demonstrated no significant differences in infection prevalence or intensity between February and April. Thus, conclusions concerning comparisons of infection prevalence and intensity between 2002 and 2005 and among locations and sites sampled during different months in 2005 are not confounded. Of course, if sampling were done outside the summer – autumn period in which water temperature peaks, then decreased prevalence and intensity of infections might be expected. Seasonal and annual differences in water temperature along the NSW coast and consequences for *Perkinsus* sp. infections in abalone are discussed in detail in Chapter 6.

The broad-scale surveys done in 2002 and 2005 provide information about the prevalence and intensity of *Perkinsus* sp. infections at scales of several – 100's of km. They have provided no information about the prevalence and intensity of infections at scales of metres or 100's of metres. The pilot survey of *Perkinsus* sp. infections in abalone in 5 areas within a 1 km length of coastline around Beecroft Head at "North Jervis Bay" provided information at the scale of 100's of metres. Prevalence and intensity of *Perkinsus* sp. infections did not differ significantly among these areas. However, to make the conclusion that abalone on adjacent areas of reef (separated by 100's of metres) experience similar rates of infection and intensity requires similar sampling around multiple sites. This was beyond the scope of this project. The pilot study did, however, highlight the need for alternative experimental designs for sampling sparse abalone populations to estimate prevalence of *Perkinsus* sp. at small spatial scales (100's of metres or less). Finer-scale sampling in the vicinity of fronts of infection and disease would be fundamental to understanding mechanisms of *Perkinsus* sp. transmission and frontal progression of perkinsosis.

4.5. Conclusions

- Ray's test on samples of gill tissue was the most sensitive diagnostic method for the detection of *Perkinsus*. Histology (on multiple organs/tissues) detected only 33% of the infections detected by Ray's test. PCR detected only 54% of the infections detected by Ray's test.

- *P. olseni* was positively identified, by PCR and gene sequencing, as one species of the *Perkinsus* genus infecting blacklip abalone in NSW.

- Identification, by PCR and gene sequencing, of a *Perkinsus* sp. with a previously unknown ITS-region gene sequence (*Perkinsus* sp.-variant ITS) suggests the presence of a variant strain of *P. olseni,* or alternatively, a previously undescribed species.

- In 2005, *Perkinsus* sp. infections in abalone were identified at multiple survey sites, within multiple survey locations between Point Perpendicular (near Laurieton and within location "North Coast") on the north coast of NSW and St. Georges Head (within location "South Jervis Bay). *Perkinsus* sp. was also identified at a single isolated site (Merimbula, within location "Tathra-Eden") on the far south coast of NSW.

- *P. olseni* was positively identified at multiple survey sites within multiple survey locations between Point Perpendicular (within location "North Coast") and St. Georges Head (within location "South Jervis Bay").

- *Perkinsus* sp.-variant ITS was positively identified at multiple survey sites within multiple survey locations between the northern side of Crowdy Head (within location "Crowdy Head") and St. Georges Head (location "South Jervis Bay") and at a single isolated site (Merimbula, within location "Tathra-Eden") on the far south coast of NSW.

- Detection of *Perkinsus* sp. infections in abalone during 2005, at locations to the north and south of the previously documented range of *Perkinsus*, represents an extension in the documented geographic range of *Perkinsus* sp. infections in blacklip abalone in NSW. Actual extension of geographic range since 2002 cannot be concluded because the northern and southern boundaries of distribution identified in 2005 were outside the geographic range of the 2002 survey.

- Prevalence of *Perkinsus* sp. infections in abalone in 2005 differed significantly among locations and among sites within locations.

- Prevalence of infections during 2005 was greatest in populations of abalone at the northern and southern margins of the infected zone between locations "North Coast" and "South Jervis Bay" (excluding the "outpost" of infection at Merimbula). This is consistent with maximum prevalence occurring at the boundaries of the infected zone (locations "Port Stephens" and "South Jervis Bay") during 2002.

- Intensity of *Perkinsus* sp. infections in abalone (based on Ray's test grade) differed significantly among locations in 2005.

- Although there was no significant difference in the prevalence of *Perkinsus* sp. infections in abalone in 2005 compared to 2002, intensity of infections was significantly greater in 2002 (Ray's test grade: 1 – 3) compared to 2005 (Ray's test grade: 0.5 – 4).

- The greater intensity of *Perkinsus* sp. infections in 2002 compared to 2005 explains the better relative sensitivity of Ray's test and histology in 2002 compared to 2005. This is because the relative sensitivity of these diagnostic methods is better for high-grade infections (96% for Ray's grade ≥ 2 in 2005) than low-grade infections (8% for Ray's grade < 2).

5. PATHOGENESIS OF *PERKINSUS SP.* IN ABALONE IN NSW

5.1. *Introduction*

Perkinsus spp. have been demonstrated as primary pathogens and are responsible for, or associated with, mass mortalities of a wide variety of molluscan hosts throughout the world (Villalba *et al.*, 2004). Consequently, infections with *P. marinus* or *P. olseni* are listed as notifiable diseases of molluscs by the OIE (OIE, 2009). *P. olseni* (and con-specific *P. atlanticus*) and a search of published literature indicates that this species has been implicated or associated with mortalities in wild or cultured stocks of mollusc species in Australasia (e.g., Lester, 1986; Lester *et al.*, 1990; Goggin and Lester, 1995; OIE, 2009), SE Asia (e.g., Park *et al.*, 2005; OIE, 2009), and Europe (Azevedo, 1989; Villalba *et al.*, 2005; OIE, 2009).

In Australia, *Perkinsus* has been identified in a wide variety of shellfish (Goggin and Lester, 1987, 1995; Hine and Thorne, 2000). Despite the identification of *Perkinsus* infecting a moribund *Tridacna gigas* (Goggin and Lester, 1987) and mass mortalities of this species of giant clam in northern Queensland (Alder *et al.*, 1986), the contribution of *Perkinsus* sp. to the mortalities is unclear. *Perkinsus* has been associated with mortalities of greenlip abalone, in South Australia (Lester, 1986; Lester *et al.*, 1990; Goggin and Lester, 1995), and more recently blacklip abalone in New South Wales (see Chapter 2). In South Australia, *P. olseni* was first detected in blacklip abalone in the Spencer Gulf in the late 1970's (Lester and Davis, 1981) and was later associated with mortality of greenlip abalone in Gulf St Vincent in 1980 and each summer from 1982 – 1985 (Lewis *et al.*, 1987; Lester *et al.*, 1990; O'Donoghue *et al.*, 1991). Diseased blacklip abalone had been noticed since 1972 by abalone divers, who observed yellowish pustules in the flesh of the abalone (Lester and Davis, 1981). Clusters of *P. olseni* cells were also found in fresh smears of the haemolymph (Lester and Davis, 1981). In 1986 a survey of greenlip abalone in die-back areas identified heavily infected abalone without macroscopic signs such as pustules and it was not possible to distinguish between heavily infected and uninfected individuals in the field (Lester, 1986). However, in a later survey *P. olseni* infections in both blacklip and greenlip abalone were characterised by macroscopic necrotic nodules (0.5 – 8 mm diameter) on the surfaces of muscles and mantle, and in some abalone the nodules had developed into abscesses containing creamy viscous fluid (O'Donoghue *et al.*, 1991). The nodules showed inclusions of variable numbers of inflammatory cells (amoebocytes), other host cells, and multiple life-stages of *P. olseni* (trophozoites, dividing stages or tomonts and merozoites), including both live and dead parasites (O'Donoghue *et al.*, 1991; Goggin and Lester, 1995). Prezoosporangia were observed in nodules in the tissue of blacklip abalone but not in greenlip abalone (O'Donoghue *et al.*, 1991).

The study by O'Donoghue *et al.* (1991) concluded that *P. olseni* was pathogenic and caused necrotic lesions within abalone tissues. Moribund greenlip abalone in Gulf St Vincent were heavily infected with *P. olseni* (Lester, 1986) and surveys showed a strong correlation between the presence of *P. olseni* infection and areas of die-back of greenlip abalone (Lester *et al.*, 1990; O'Donoghue *et al.*, 1991). Although toxicology studies ruled out some of the main chemical pollutants as a cause of the mortalities, other causal factors could not be ruled out (Shepherd, 1985; Lester *et al.*, 1990; O'Donoghue *et al.*, 1991). Blacklip abalone were also infected by *P. olseni* but reports of mass mortalities of this species in the wild were lacking (Lester *et al.*, 1990). Thus a direct link between mortality of wild abalone and *P. olseni* was not established.

Importantly, evidence that *P. olseni* is capable of inducing disease and mortality in South Australian greenlip abalone was provided via a controlled laboratory experiment (Goggin and Lester, 1995). Hatchery-reared greenlip abalone were exposed to zoospores of *P. olseni* and held at 2 different temperatures, 15°C and 20°C. Within 2 months, 49 of the 53 experimental abalone were dead (23/23 at 15°C and 26/30 at 20°C) compared to only 9 out of 53 dead in the control groups (3/23 at 15°C and 6/30 at 20°C). Post-mortem application of Ray's test confirmed *P. olseni* infections in the experimental abalone and the absence of infections in the control abalone (Goggin and Lester, 1995). Goggin and Lester (1995) also report another laboratory experiment, previously unpublished by Lester, involving blacklip abalone. Although this experiment was uncontrolled, one group of abalone, held in water of 15°C, developed abscesses and *P. olseni* within these abscesses were dead. In another group of 4 abalone, held at 15°C and subsequently 20°C, one specimen died and live *P. olseni* were recovered from the nodules and haemolymph of the remaining 3. Further evidence of *P. olseni* pathogenicity is the mortality events of greenlip abalone within culture facilities that occurred following the introduction of blacklip specimens from *P olseni* endemic areas into the tanks (Goggin and Lester, 1995).

Despite the uncertainty about whether *P. olseni* was a primary cause of observed mortalities of wild greenlip abalone in South Australian waters, the necrotic lesions within host tissues clearly demonstrated *P. olseni* as a pathogen in wild greenlip and blacklip abalone. The most convincing of the laboratory experiments (by Goggin, 1990, cited in Goggin and Lester, 1995) demonstrated the mortality of greenlip abalone due to *P. olseni* under laboratory conditions. Similarly, mortality of giant clams, *T. gigas*, following their exposure to *Perkinsus* sp. parasites was demonstrated in the laboratory (Unpubl. data referred to in Goggin, 1996). The result provided more certainty about the pathogenicity of *Perkinsus* sp. to this clam species. Prior to the experiments *Perkinsus* sp. was associated with mass mortalities of *T. gigas* on the Great Barrier Reef on the basis that moribund clams were found to be heavily infected with the parasite (Goggin and Lester, 1987) but other explanations for the clam mortalities such as environmental variables, although considered unlikely, could not be ruled out (see Alder *et al.*, 1986).

In NSW, *Perkinsus* sp. has been associated with mortality events (see Chapter 2) and the pathology of perkinsosis has been described in abalone that were alive prior to being sampled in 2002-03 for the NSW component of the National Survey of Diseases in Abalone (Callinan and Landos, 2006). Histological examinations of moribund abalone have been limited to the few individuals collected following mortality events (Chapter 3). In NSW, presentation of perkinsosis in live blacklip abalone differs from that described in South Australia. External macroscopic nodules and abscesses have not been observed in any NSW abalone that were alive at the time of sampling (Callinan and Landos, 2006). The pathology also differed from that described for blacklip abalone from South Australia (Lester and Davis, 1981; O'Donoghue *et al.*, 1991; Goggin and Lester, 1995), with NSW blacklip abalone apparently unable to wall off and contain *Perkinsus* sp. cells, probably resulting in their mortality (Callinan and Landos, 2006; Handlinger *et al.*, 2006). We note that at the time of die-backs in South Australia, greenlip abalone also did not show signs of long-term infection, such as the abscesses seen in blacklip abalone in South Australia (Lester, 1986). The NSW component of the NSDA included observations of pathology in 16 abalone in which *Perkinsus* sp. was detected by histology. Trophozoites and tomonts were identified in multiple tissues and in most cases, were associated with significant tissue necrosis and infiltrations of haemocytes, signifying a host response to infection. *Perkinsus* sp. infected haemolymph vessels, gills, sub-enteric haemocyte beds, the right kidney, gonad, hypobranchial gland and the foot muscle. The most commonly affected area appeared to be the haemocyte beds beneath the mucosa of the gut (Callinan and Landos, 2006).

Differences in the pathology of *Perkinsus* sp. and *P. olseni* infections in blacklip abalone in NSW compared to South Australia suggest differences in pathogenicity of the parasite (Handlinger *et al.*, 2006). Pathogenicity of *Perkinsus* spp. in mollusc hosts or more generally, of any pathogen in any host, is a function of the virulence of the pathogen, mechanisms of host response and interactions

of these factors with environmental variables. Moreover, virulence of pathogens and effectiveness of host response may vary among genetic strains of the pathogen and host. For example, amongst *Perkinsus* spp., genetic strain variation has been most thoroughly studied in *P. marinus* and Reece *et al.* (2001) identified 12 different genetic strains along the Atlantic and Gulf coasts of the USA. In addition to the non-uniform geographic distribution of these 12 strains, there was evidence that oysters could be infected by multiple genetic strains of *P. marinus* simultaneously. There has been no equivalent study of intra-specific genetic variation in *P. olseni* in Australia.

Conclusive evidence for the primary portals of entry for *Perkinsus* parasites into hosts is lacking (Villalba *et al.*, 2004) but results from experimental infections of hosts (e.g., Chintala *et al.*, 2002) and identification of organs infected in hosts with low-intensity infections (e.g., Casas, 2002 cited in Villalba *et al.*, 2004) note the importance of the gill and labial palps as primary portals of entry for clams. For oysters, that gill, labial palps and mantle are equally, if not more, important than gut epithelium (Chintala *et al.*, 2002). Subsequent multiplication of the parasite and progression into multiple organs in the host results in destruction of tissue, loss of normal structure, probable organ dysfunction and may lead to death of the host (e.g., La Peyre *et al.*, 1995; Villalba *et al.*, 2004; Park *et al.*, 2005).

In addition to mortality, *Perkinsus* parasites can have sub-lethal effects on hosts that may have important ecological and economic implications. These sub-lethal effects include: negative effects on growth and condition of hosts (e.g., Andrews, 1961; Casas, 2002, cited in Villalba *et al.*, 2004; Brown *et al.*, 2005); reduced reproductive potential due to inhibition of gametogenesis, decreased spawning frequency and egg production (e.g., Dittman *et al.*, 2001; Casas, 2002, cited in Villalba *et al.*, 2004; Park *et al.*, 2006a); and increased susceptibility to secondary infections (e.g., Montes *et al.*, 2001). Such effects are the likely consequence of a general weakening of the host due to decreases in the energy available for investment in growth, reproduction and immune responses. Results of studies of the balance between energy acquisition and consumption have been variable (Villalba *et al.*, 2004) but some predict negative energy balances due to *Perkinsus* infections (e.g., Choi *et al.*, 1989; Casas, 2002, cited in Villalba *et al.*, 2004).

An overview of the pathogenesis of infections of *Perkinsus* spp. in abalone and subsequent host responses is provided by Villalba *et al.* (2004). Hooper *et al.* (2007) provide a review of stress and immune responses in abalone. The primary host response to *Perkinsus* infection in abalone species, venerid clams and several other species involves infiltration of haemocytes into tissues and encapsulation of parasite cells by the haemocytes (see review by Villalba *et al.*, 2004; and Cremonte *et al.*, 2005; Park *et al.*, 2005). Intense infections are characterised by multiple large foci where both free and encapsulated parasites occur. Typically, these inflammatory foci become macroscopically evident as pustules or abscesses. Associated with encapsulation, secretion by haemocytes of polypeptides, likely to destroy the parasite, has been described in several instances (Chagot *et al.*, 1987; Montes *et al.*, 1995; Sagristà *et al.*, 1995).

A range of environmental factors affect pathogenicity of *Perkinsus* spp., either by affecting the virulence of the parasite, the capacity of the host to respond to infection or their interaction (Villalba *et al.*, 2004). The role of environmental factors (e.g., water temperature, salinity, pollution and food availability) in the epizootiology of perkinsosis is considered in Chapters 6. It is relevant here, however, to note the evidence for these factors affecting the intensity of *Perkinsus* infections in hosts. Intensity of *P. marinus* infections increases with increasing salinity and temperature. A seasonal pattern in the intensity of *P. marinus* infections in oysters (Villalba *et al.*, 2004) and *P. olseni* / *P. atlanticus* infections in clams (Villalba *et al.*, 2005) is determined by the annual temperature cycle, with intensity lowest in winter months, increasing in spring and peaking in late summer/autumn at which time mortalities are most concentrated. *In vivo* and *in vitro* laboratory experiments have also demonstrated the influence of temperature and salinity on disease progression and regression by modulating the host immune system (e.g., Chu and La Peyre, 1993a,

1993b) as well as *Perkinsus* activity (e.g., Auzoux-Bordenave *et al.*, 1995; Ahn and Kim, 2001; Casas *et al.*, 2002).

Investigations of the pathogenicity of *Perkinsus* sp. in blacklip abalone on the NSW coast were limited, in the current study, to analyses based on the pathology of infections observed by histopathology using tissue samples derived from live abalone that did not exhibit overt signs of infection. The results were compared with Ray's grading of infection intensity to assist with our interpretation of level of *Perkinsus* sp. infection with respect to disease progression and mortality (e.g., Brousseau and Baglivo, 2000). An understanding of pathogenesis of *Pekinsus* sp. to NSW abalone is essential for our interpretation of the *Perkinsus*-related mortality events that have been documented for NSW (Chapter 2). The principal objectives of this component of the project, presented in this chapter, were to:

(i) investigate the distribution of P*erkinsus* sp. infections in organs/tissues of blacklip abalone;

(ii) investigate the pathology and pathogenicity of *Perkinsus* sp. (tissue necrosis, changes in organ morphology and likely functional impairment of organs, host-response to infection);

(iii) identify pathogens other than *Perkinsus* sp. (and describe associated pathology and pathogenicity if detected);

(iv) assess the effect of *Perkinsus* sp. infection on the condition (weight-length relationship) of abalone;

(v) assess evidence regarding the site (organ/ tissue) of initial infection and disease progression (based on the distribution of *Perkinsus* sp. in organs/ tissues from (i)).

In addressing these objectives, conclusions were also made with respect to *P. olseni* and *Perkinsus* sp.-var. ITS (as distinct from *Perkinsus* sp.) for the small subset of abalone for which specific identification was made by PCR and gene sequencing.

5.2. Methods

5.2.1. Histology

Histology was examined for a sub-set of abalone (n = 209) collected during the 2005 survey. These abalone were sourced from both the broad-scale survey and auxiliary surveys described in Chapter 4. This sub-set included a total of 87 abalone that tested positive for suspected-*Perkinsus* by Ray's test (Grade 0.5 = 30, Grade 1 = 32, Grade 2 = 22, and Grade 3 = 3). The general methods for histological examination were fully described in Chapter 4 (Sections 4.2.1.2 and 4.2.1.3). Five standardised ~ 5 mm partial cross-sections of tissue were taken from preserved whole abalone and processed using standard histological techniques. The sections included foot muscle, epipodium, oesophagus (where possible), digestive gland and hind-gut, kidney, heart (where possible), gills, and gonad from each individual. Tissue sections were cut at 2 – 5 µm and stained with haematoxylin and eosin. Each organ was examined *in situ*, preserving the relationship to surrounding organs. Stained tissue sections of the abalone were examined using light microscopy and all parasites, abnormalities, and potential pathogens that were observed were recorded for each individual.

For each tissue or organ examined the number of parasite cells observed was categorised as either 1 (1 – 5 cells), 2 (6 – 10 cells), or 3 (> 10 cells). Trophozoite and dividing (tomont) life-stages of *Perkinsus* sp. were not differentiated from one another and each was counted as single parasite

cell. Gregarine parasites were usually present in the extensive hemocoel of abalone, thus their position with respect to surrounding organs and other tissues was recorded. Presence of amoeboid protozoa within organs and tissues was noted but the organisms were not counted.

For each abalone examined, the pathology of disease such as changes induced in the morphology of organs and tissues by infection was described. Response of the host abalone to infection was also measured by scoring haemocyte activity (prevalence, aggregations) on a scale of increasing abundance from 1 (few), 2 (medium), to 3 (many). Note that in the current report, we do not distinguish among types and sub-types of haemocytes (the approach adopted by Villalba *et al.*, 2004). Fisher's Exact test was used to test for significant differences in the frequency of the grades of haemocyte activity among the grades of infection intensity (Ray's test grades).

5.2.2. Effect of Perkinsus sp. *infection on condition of abalone*

To test whether the "condition" (relationship between weight and length) of abalone was affected by *Perkinsus* sp. infection, analysis of covariance (ANCOVA) was used with a sub-set of data collected from abalone at sites near Jervis Bay. Shell lengths (to the nearest mm) and wet meat weights (to the nearest gram) of 212 abalone collected from 3 sites (Beecroft Head, Targets, St. Georges Head) within the locations "North Jervis Bay" and "South Jervis Bay" were used for this analysis. Relationships between wet meat weight and length were made linear by log-transformation of both variables. ANCOVA was used to test for differences in the relationship between log(meat weight) and log(length) of abalone in 3 groups: no *Perkinsus* sp. infection; low-grade (Ray's test grades 0.5 and 1); and high-grade (Ray's test grades ≥ 2) infections.

This sub-set of data from the 3 sites described above comprised abalone sampled from these sites during the broad-scale survey and auxiliary surveys described in Chapter 4. This analysis was restricted to abalone from these 3 sites because: (i) proportions of abalone infected with *Perkinsus* sp. at these sites were relatively high; and (ii) numbers of abalone with high-grade infections were greatest at these sites; and (iii) these sites are close together on the coast of NSW. If abalone from all sites on the NSW coast had been included and there was a difference in length-weight relationships among sites (associated with latitude), then this would bias estimates of length-weight relationships for abalone with no infection or low-grade infections relative to high-grade infections (only 3 high-grade infections were found at sites other than North Jervis Bay or South Jervis Bay).

5.3. Results

5.3.1. Distribution of Perkinsus sp. and P. olseni in organs

A total of 29 *Perkinsus* sp. infections were detected in the 209 abalone examined using histology. Intensity of infection (based on numbers of *Perkinsus* sp. cells observed) was not recorded for 2 of these abalone (only presence / absence was recorded) so these data were excluded from the following analyses. Thus, the following analyses comprise a sample size of 27 abalone.

Of the 29 *Perkinsus* sp. infections identified by histology, positive identification by PCR and gene sequencing of *P. olseni* was also made in 5 cases and there was no positive identification of *Perkinsus* sp.-variant ITS. Of the 27 *Perkinsus* sp. infections identified by histology for which infection intensity was also recorded, *P olseni* was identified by PCR and gene sequencing in 4 cases.

The incidence of *Perkinsus* sp. infections differed among organs: Intestine (21/27, 78%), gills (18/27, 67%), kidneys (16/27, 59%), stomach (13/23, 57%) and heart (7/15, 47%) were infected most frequently (Fig. 5.1). Less frequently affected were epipodium (10/23, 43%), digestive gland

(8/27, 30%) and oesophagus (4/23, 17%). Muscle (2/27, 7%) and haemolymph within the hemocoel (1/23, 4%) were infected infrequently and no infections of *Perkinsus* sp. were observed in gonads (0/26, 0%) (Fig. 5.1).

For the 5 cases of *P. olseni* infection identified by PCR and gene sequencing,, incidence of infections observed in specific organs by histology were: Intestine (2/5), gills (4/5), kidneys (3/5), stomach (1/5), heart (1/2), epipodium (1/5), digestive gland (1/5), oesophagus (0/5), muscle (2/5), haemolymph within the hemocoel (1/5) and in gonads (0/4).

The frequency of each of 3 grades of infection intensity (Grade 1 for 1 – 5 cells, Grade-2 for 6 – 10 cells, Grade-3 for > 10 cells present) is also shown in Figure 5.1. Histology could not be assessed for every organ in each individual abalone as sections from individual animals sometimes missed target organs. A sub-set of 5 organs (kidneys, gills, intestine, muscle and digestive gland) were examined for all 27 abalone. For these 5 organs, there was no apparent difference in the ratios of Grade 1, Grade 2 and Grade 3 infections.

Perkinsus sp. infections were generally observed in multiple organs/ tissues of individual abalone. In only 6 of the 27 abalone was a single organ or tissue infected. In 17 of 27 abalone, 3 or a greater numbers of organs/ tissues were infected with *Perkinsus* sp. Median number of organs/ tissues infected was 4 (range 1 – 8). Thus, infections detected in these 27 abalone were generally systemic. Conclusions of systemic infection and the range of organs and tissues infected in abalone sampled in 2002-03 were similar (Callinan and Landos, 2006). In contrast to the 2005 survey, *Perkinsus* sp. infections were found in gonad tissue of abalone sampled in 2002-03.

For the 5 cases of *P. olseni* infection identified by PCR and gene sequencing, 3 or a greater number of organs/tissues were infected with *Perkinsus* in 4 of the 5 abalone. The median number of organs/tissues infected was 3 (range 2 – 5). Consistent with the results for *Perkinsus* sp., infections were systemic.

It is also of interest that in the 6 instances that infection was detected in a single organ, the organ affected was the gill in 4 abalone, and epipodium and stomach in each of another 2 abalone. Intestine, the organ most frequently infected in the 27 abalone examined here, was not the site of infection in any of the 6 abalone in which only a single organ was infected.

5.3.2. Evidence of organ destruction and impairment

There was no histological evidence of organ destruction or impairment in abalone not infected with *Perkinsus* sp. This was in marked contrast to the pathology associated with abalone heavily infected with *Perkinsus* sp. Necrotic tissue associated with *Perkinsus* sp. was observed in the digestive gland of some individuals. Inflammation was noted in the wall of the intestine (with associated ulceration), stomach and gills of several abalone, including one of the abalone for which *P. olseni* was positively identified by PCR. The most striking and general observation was of significant aggregation and infiltration of haemocytes into tissues (see Section 5.3.3).

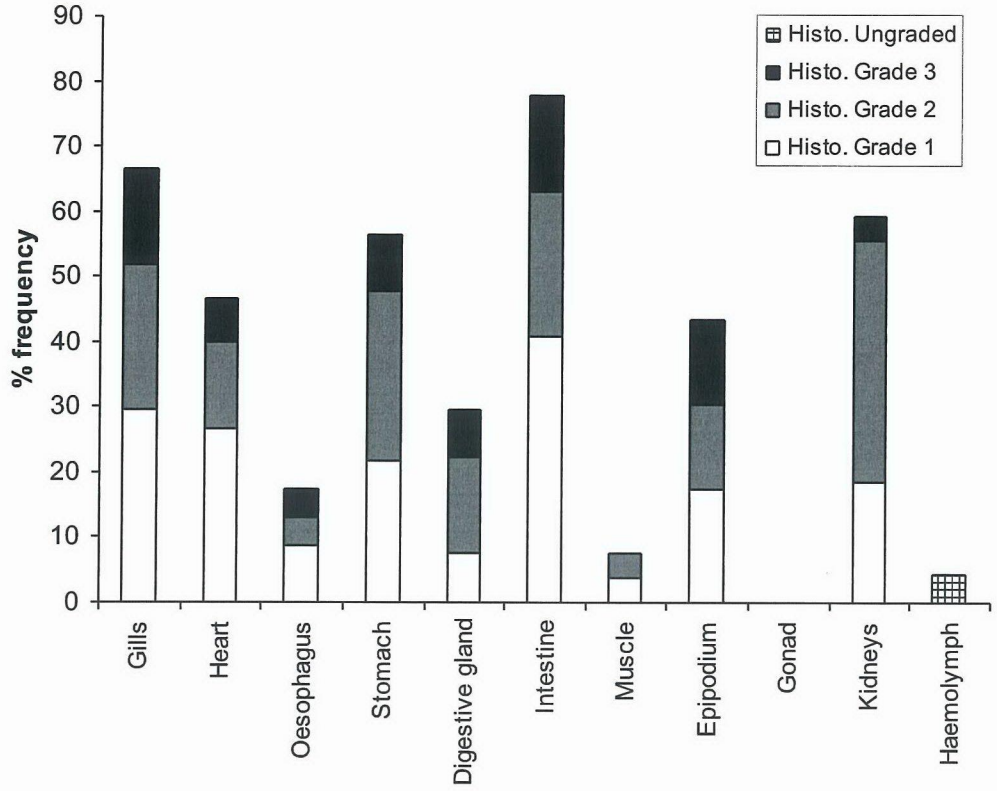

Figure 5.1. Frequency of *Perkinsus* sp. infection, and of 3 grades of infection intensity (increasing in level of intensity from 1 to 3), determined by histology.

Overall, the pathology observed in abalone sampled during 2005 was less severe than observed in the 2002-03 sample of abalone. Tissue necrosis was more extensive in abalone from the 2002-03 survey (Callinan and Landos, 2006). With respect to the haemocyte beds beneath the mucosa of the gut Callinan and Landos (2006) observed: *"Infections at this site had led to disruption of the gut epithelium and sloughing of mucosa and Perkinsus organisms into the lumen"*. And, *"... proliferation of Perkinsus organisms within gill vessels sometimes resulted in vessel blockage (infarction) and gill necrosis."* Several images of the pathology observed from 2002-03 samples and published in Callinan and Landos (2006) are reprinted here in Appendix 5 – Section 12.5.2.

Based on grades of infection intensity in gill tissue from Ray's test, the 16 abalone sampled during 2002-03 that tested positive to the presence of *Perkinsus* sp. by histology, were more severely infected than the sample of 29 abalone from 2005. Grades of infection from Ray's test ranged from 1 – 5 (median Grade 3) for the 2002-03 sample but only between 0.5 – 3 (median Grade 2) for the 2005 sample. The most extreme pathology observed in abalone tissues from 2002-03 was observed in tissue samples from abalone that were Grade 4 and Grade 5 by Ray's test on gill tissue. Thus, the greater intensity of infections observed in the 2002-03 compared to the 2005 sample (based on Ray's test grades) corresponds with the greater tissue degeneration and necrosis observed in the 2002-03 sample.

The pathology associated with *Perkinsus* sp. observed in 16 abalone from 2002-03 and now 29 abalone from 2005, 45 abalone in total, provides solid evidence of *Perkinsus* sp. as a pathogen in blacklip abalone in NSW. More specifically, pathology associated with the 5 specimens from 2005, within which *P. olseni* was positively identified by PCR, provides solid evidence of *P.*

olseni as a pathogen in blacklip abalone in NSW. Because there was no observation of *Perkinsus* by histology for any of the 5 abalone identified by PCR and gene sequencing as having *Perkinsus* sp.-variant ITS infections, the pathogenicity of *Perkinsus* sp.-variant ITS remains unresolved.

5.3.3. Host response to infection

Upon examination, the amount of haemocyte activity and aggregation within organ tissue was graded qualitatively (by the same observer, P. Gill) into four categories: "-" indicating no activity and "+", "++" and "+++" to indicate increasing activity and aggregation of haemocytes in organ tissues. Proportions of the 4 grades of haemocyte activity differed significantly among abalone classified by infection intensity (Ray's test grade) (Fisher's Exact test, $P < 0.01$). Tissue infiltration and aggregation of haemocytes was greatest in abalone with higher grade infections (Fig. 5.2) – evidence of a host response to infection that increases with infection intensity.

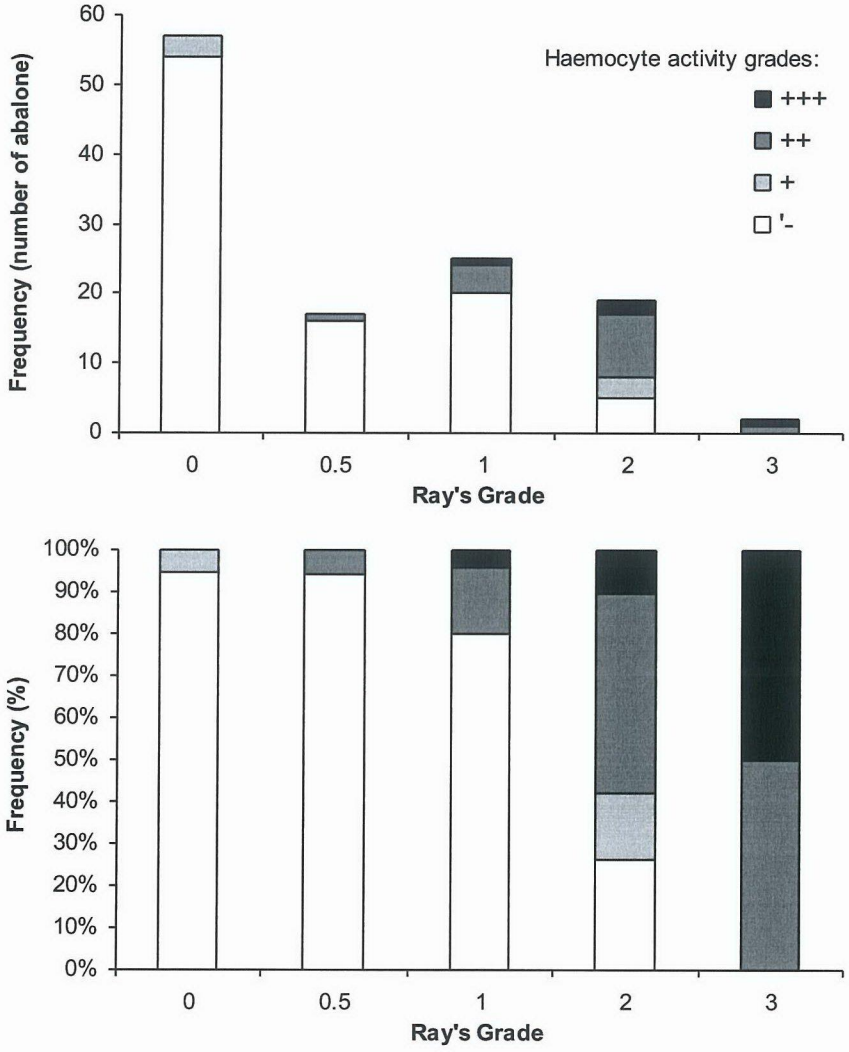

Figure 5.2. Relationship between intensity of infection (based on grades of Ray's test on gill tissue) and haemocyte activity (based on 4 grades from histology) in 209 abalone. Upper panel represents absolute frequency. Lower panel represents relative frequency.

For the 4 cases of *P. olseni* infection identified by PCR and gene sequencing, for which the amount of haemocyte activity and aggregation within organ tissue was recorded, activity was noted in all cases (2 of "+" and 2 of "++").

5.3.4. Effect of Perkinsus sp. infection on condition of abalone

There was no significant difference in the condition (length – meat weight relationship) of abalone with low-grade infections (Ray's grades 0.5 and 1), high-grade infections (Ray's grades ≥ 2) or no infection (Fig. 5.3 and Table 5.1).

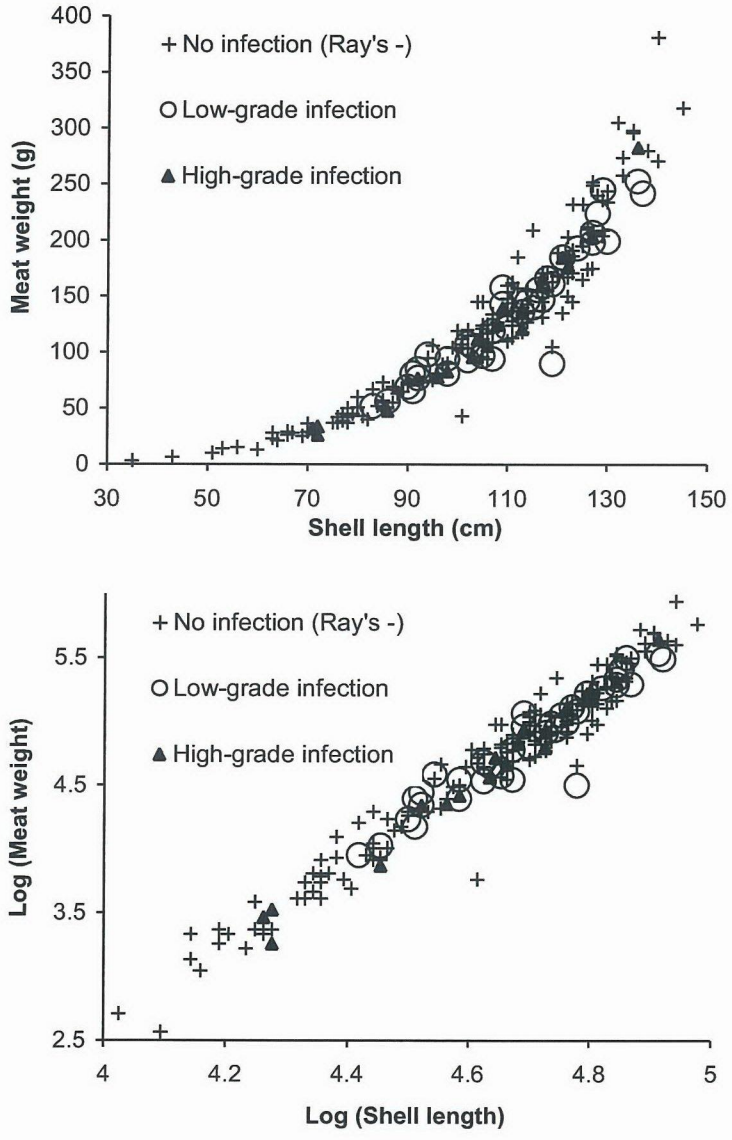

Figure 5.3. Relative condition of abalone with high-grade, low-grade and no infections of *Perkinsus* sp. – meat weight versus shell length and log(meat weight) versus log(shell length).

Table 5.1. ANCOVA summary table, log (Meat weight) v Log (Shell length).

Treatment	Intercept	Slope	SS	df	MS	F	P
No infection	-10.474	3.266	3.5057	154	0.0228		
Low-grade infection	-9.352	3.021	0.6804	34	0.0200		
High-grade infection	-11.220	3.420	0.1414	18	0.0079		
Summed			4.3276	206	0.0210		
Common	-10.473	3.261	4.3823	208	0.0211		
Difference among slopes			0.0548	2	0.0274	1.304	0.274 NS
Total	-10.427	3.254	4.4215	210	0.0211		
Difference among adjusted means			0.0392	2	0.0196	0.929	0.397 NS

5.3.5. Presence of other pathogens and organisms

Apart from *Perkinsus* sp., no other pathogens were detected by histological examination. Other organisms found in more than one abalone were gregarine protozoa (in 54/209 abalone), mostly in the hemocoel and associated with the intestine, and incidental amoeboid protozoa (in 36/209 abalone), which were found mostly in the gills. There was no significant difference in the frequency of gregarines or amoebas detected in abalone between groups that were or were not infected with *Perkinsus* sp. (Gregarines: Chi-squared = 1.298, $df = 1$, $P = 0.254$; Amoeba: Chi-squared = 0.278, $df = 1$, $P = 0.598$). Thus, host response (haemocyte activity) to *Perkinsus* sp. was not confounded by significant differences in the incidence of other organisms present in abalone.

Perkinsus sp. (with specific identification of *P. olseni* by PCR in several cases) was the only pathogen identified in any of the 209 abalone examined in this study. Similarly, *Perkinsus* sp. was the only pathogen found in any of the 407 wild abalone examined histologically in 2002-03 (n = 380 published in Callinan and Landos, 2006; n = 27 from 2002-03 NSDA data-set but not published).

5.4. Discussion

In this study, observations of the pathology of *Perkinsus* sp. infections were made in 29 blacklip abalone. These observations, when combined with information gained from the 16 abalone in which *Perkinsus* sp. was identified by histology during the NSDA, inform several significant conclusions about the pathogenicity of *Perkinsus* sp. parasites (including *P. olseni*) in blacklip abalone in NSW.

Many more abalone than those described above were examined using histology: a total of 209 from the 2005 survey and 407 wild abalone sampled during 2002-03 for the NSDA. No pathogens other than *Perkinsus* sp. (and *P. olseni*) were identified in either of these surveys. Nor were any other pathogens identified upon examination of abalone associated with mortality events during the 1990's (Table 3.1 and Appendix 4 in Section 12.4). There is no evidence of any pathogen, other than *Perkinsus* sp. (and *P. olseni*), in populations of blacklip abalone in NSW during the past 15 years. Moreover, the evidence of disease in abalone sampled during 2002 and 2005 including tissue necrosis, inflammation and haemocyte infiltration and aggregation cannot be associated with any organism other than *Perkinsus* sp. (and *P. osleni*).

Infections of *Perkinsus* sp. that were identified by histology were generally in tissue samples from abalone that were diagnosed with high-grade infections (> 100 cells) by Ray's test (see Section 4.3.1). Moreover, infections detected by histology were generally systemic. In 4 of the 6 instances that histology detected *Perkinsus* sp. parasites in a single organ, the infected organ was the gill. Whilst this observation is consistent with the gill being the primary portal of entry for *Perkinsus*

sp. it does not demonstrate this. Better evidence would come from a more sensitive methodology, applied to tissue from multiple organs from a sample of abalone. If, using such a methodology, gill tissue was consistently the only site of infection in abalone with low-intensity non-systemic infections, then the evidence for gill as the primary portal of entry for *Perkinsus* sp. into blacklip abalone would be substantial. A PCR assay, ideally quantitative (such as Taqman) with sufficient sensitivity, or other advanced molecular techniques such as immunoassays (e.g., Remacha-Trivino *et al.*, 2008), would facilitate such analyses. Alternatively, Ray's test could potentially be applied to multiple organs/tissues. Laboratory experiments, where uninfected abalone are exposed to infective stages of *Perkinsus* sp. and then sampled over time, could also be done to investigate primary site(s) of parasite entry into the abalone and disease progression.

The result that infections detected by histology were generally systemic is consistent with conclusions drawn from the 2002-03 survey (Callinan and Landos, 2006). Indeed, the proliferation of *Perkinsus* spp. and progression into multiple organs is well described in other hosts (Villalba *et al.*, 2004, Park *et al.*, 2005).

In abalone with high-intensity infections, there was evidence of substantial tissue and organ damage. Necrotic tissue associated with *Perkinsus* sp. trophozoites and tomonts was observed in abalone sampled during 2003 and 2005. The extensive damage to tissues observed in the most severely infected abalone from 2003, disruption of the gut epithelium and infarction in the gills, suggests impairment to normal nutrient absorption and respiration. The abalone from which these tissues were sampled were alive, but moribund, when collected and, given the severity of the lesions observed histologically, it is likely that such intense infections would result in mortality. Histopathology done on moribund abalone, sampled from a future mortality event, would provide further insights into the degree of tissue necrosis and destruction associated with morbidity and mortality (see Section 7.3).

Infiltration and aggregation of haemocytes were observed in tissues from samples of abalone from 2003 and 2005. For the 2005 sample, a qualitative grading of haemocyte activity and aggregation within organ tissue demonstrated a positive relationship between infection intensity (Ray's test grade) and haemocyte activity. Tissue infiltration and aggregation of haemocytes was greatest in abalone with higher grade (Ray's grade ≥ 2) infections. Noticeable histopathological effects in abalone at known levels of infection intensity, estimated by Ray's test, allows interpretation of level of *Perkinsus* infection with respect to disease progression and mortality (e.g., Brousseau and Baglivo, 2000). The lack of encapsulation of *Perkinsus* sp. cells by haemocytes, as described for *P. olseni* infections in South Australian blacklip abalone (Lester, 1986), suggests a difference in the virulence of *P. olseni* (or strain) and/or a difference in the resistance of blacklip abalone in NSW compared to South Australia. Potentially, multiple strains of *P. olseni* may exist in Australia as occurs with *P. marinus* in the USA (Reece *et al.*, 2001). The value of this line of research is discussed in Section 7.3.

The pathology associated with *Perkinsus* sp. observed in 16 abalone from 2002 and now 29 abalone from 2005, 45 abalone in total, provides solid evidence of *Perkinsus* sp. as a pathogen in blacklip abalone in NSW. This conclusion is based on the firm evidence of tissue necrosis, destruction of normal tissue structure, infarction of gills and the host response of abnormal haemocyte activity in infected tissues. Moreover, the observed histopathology for the 5 specimens sampled in 2005, within which *P. olseni* was positively identified by PCR, provides solid evidence of *P. olseni* as a pathogen in blacklip abalone in NSW. The pathogenicity of *Perkinsus* sp.-variant ITS, however, remains unresolved because there were no observations of *Perkinsus* by histology for any of the 5 abalone identified by PCR and gene sequencing as having *Perkinsus* sp.-variant ITS infections.

In addition to the conclusions drawn from histopathology, this project also addressed the possibility of a sub-lethal impact of infection on the body condition of abalone. There was no

significant difference in the condition (length – meat weight relationship) of abalone without infections, low-grade or high-grade *Perkinsus* sp. infections. Conclusions from similar analyses with respect to other hosts infected by *Perkinsus* spp. are variable. Deterioration of weight at length or other indices of condition have been reported (e.g., Paynter, 1996; Casas, 2002, cited in Villalba *et al.*, 2004; Brown *et al.*, 2005) but other studies show no effect (Paynter, 1996; Cigarria *et al.* 1997; Goggin, 1996). It is possible that body condition of abalone may deteriorate when infection intensity is greater than that observed in 2005. If higher-grade infections (i.e., Ray's grade 4 or 5) are detected in future surveys or samples following observed mortality events, a similar analysis of the effect of infection intensity on body condition would be desirable. Increased susceptibility to secondary infections is another sub-lethal effect of *Perkinsus* spp. that has been observed elsewhere (e.g., Montes *et al.*, 2001). We found no evidence of other pathogens during examination of histology of samples from this project.

The scope of this project did not extend to any study of the effects of *Perkinsus* sp. infection on reproductive potential. However, the evidence of inhibition of gametogenesis, decreased spawning frequency and egg production from other studies (e.g., Dittman *et al.*, 2001; Casas, 2002, cited in Villalba *et al.*, 2004; Park *et al.*, 2006a) should not be ignored. *Perkinsus* sp. infections were found in gonad tissue of abalone sampled during 2002-03 but not in 2005. Some evaluation of the reproductive potential of the population of abalone remaining in zones F – L of the fishery (i.e., Port Stephens to Jervis Bay) would inform any assessment of stock recovery.

A more detailed analysis of the importance of water temperature in the epizootiology of perkinsosis is presented in Chapter 6. It is relevant here, however, to note correspondence between infection intensity in samples from 2002 and 2005 and water temperature during the same years. Intensity of *Perkinsus* sp. infections in abalone from the Jervis Bay region was greater in 2002 than in 2005. Water temperatures were also greater during the 9 months prior to sampling in 2002 compared to 2005. Water temperatures were above average prior to sampling in 2002 but approximately average in 2005 (see Section 6.3.8.4). This is of note here because of the consistency of this observation with the positive relationship between water temperature and infection intensity and mortality identified in studies of *Perkinsus* spp. infections in other hosts (e.g., Villalba *et al.* 2004).

5.5. Conclusions

- Infections of *Perkinsus* sp. (including a subset of cases for which *P. olseni* was positively identified by PCR and gene sequencing) in abalone that were identified by histology were generally systemic with intestine, gill, kidney, stomach and heart most frequently infected. Less frequently infected were epipodium, the digestive gland, oesophagus, muscle and haemolymph within the hemocoel. These observations were consistent between samples from 2002-03 and 2005.

- Substantial tissue and organ damage occurred in abalone with high-intensity infections. Necrotic tissue associated with *Perkinsus* sp. trophozoites and tomonts was observed in samples from 2002-03 and 2005. In the most severely infections (2002-03), disruption of the gut epithelium and infarction in the gills, suggested impairment to normal nutrient absorption and respiration (Callinan and Landos, 2006).

- Infiltration and aggregation of haemocytes provided evidence of host response to infections of Perkinsus sp. (including a subset of cases for which *P. olseni* was positively identified) and there was a positive relationship between infection intensity (Ray's test grade) and haemocyte activity.

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- The evidence of substantial tissue necrosis, organ damage and haemocyte activity associated with *Perkinsus* sp. cells in samples from both 2002-03 and 2005 provides solid evidence that *Perkinsus* sp. (and specifically *P. olseni*) is pathogenic to blacklip abalone in NSW.

- Pathogenicity of *Perkinsus* sp.-variant ITS remains unresolved.

- The lack of encapsulation of *Perkinsus* sp. cells by haemocytes, as described for *P. olseni* infections in South Australian blacklip abalone (Lester, 1986), suggests a difference in the virulence of *Perkinsus* sp. (or strain) and/or a difference in the resistance of blacklip abalone in NSW compared to South Australia.

- No pathogens other than *Perkinsus* sp. (and *P. olseni*) were identified by histology in tissue samples from the 2005 survey. Nor were any pathogens other than *Perkinsus* sp. identified in samples from the 2003-03 NSDA survey or in the limited samples associated with mortality events.

- Infection intensity did not affect the length-weight condition of abalone sampled in 2005. Because extremely high-grade infections of *Perkinsus* sp. (Ray's grade ≥ 4) were absent from samples in 2005, this conclusion is restricted to abalone with infection intensity $\leq$ Ray's grade 3.

- No conclusions about the primary portal for entry of *Perkinsus* sp. into abalone are possible. However, the finding that gill was infected in 4 of the 6 instances that histology detected *Perkinsus* sp. in a single organ, is consistent with the gill being the primary portal of entry.

6. THE EPIZOOTIOLOGY OF PERKINSOSIS AND MASS MORTALITY OF BLACKLIP ABALONE IN NSW

6.1. Introduction

In this chapter, we consider the epizootiology of perkinsosis detected in blacklip abalone and the *Perkinsus*-related mortalities of blacklip abalone that occurred at specific times since the early 1990s and at specific locations along the NSW coast. Initially we review current knowledge of the epizootiology of *Perkinsus* spp. in mollusc hosts and then consider *P. olseni* and *Perkinsus* sp. in Australia. Finally, alternative hypotheses regarding the epizootiology of perkinsosis and *Perkinsus*-related mortality of blacklip abalone in NSW are considered.

Note that the consideration of epizootiology in this chapter concerns *Perkinsus* sp. and not *P. olseni* or *Perkinsus* sp.-variant ITS separately. This is because: (i) the status of *Perkinsus* sp.-variant ITS as a separate strain of *P. olseni* or previously unidentified species of *Perkinsus* is unclear (Chapter 4); (ii) the small proportion of abalone for which *Perkinsus* sp. infections were identified by Ray's test and *P. olseni* or *Perkinsus* sp.-variant ITS were identified by PCR and gene sequencing (Chapter 4) and (iii) the unresolved pathogenicity of *Perkinsus* sp.-variant ITS (Chapter 5).

6.1.1. Epizootiology of Perkinsus spp. in mollusc hosts

Transmission

Whilst all 3 principal life-stages (trophozoite, hypnospore or prezoosporangium, zoospore; see Section 4.1.1) of *Perkinsus* spp. have been shown experimentally to cause infections in shellfish (Villalba *et al.*, 2004), the principal stage for transmission of the disease in the natural environment is unknown (Chu, 1996). Controlled laboratory infection challenges have demonstrated infection of eastern oysters, *C. virginica*, by *P. marinus* to be dose dependent (e.g., Chu and Volety, 1997). Transmission of *Perkinsus* spp. occurs both with and without alternative / intermediate hosts (e.g., White *et al.*, 1987; Goggin and Lester, 1995; Villalba *et al.*, 2004).

Both live and dead hosts are potentially sources of infective stages of *Perkinsus* spp. For example, viable *P. marinus* trophozoites are released from live *C. virginica* through diapedesis and in faeces (Scanlon *et al.*, 1997; Bushek *et al.*, 2002; Villalba *et al.*, 2004). Assuming that zoosporulation following hypnospore formation occurs in the natural environment as has been observed in vitro, then transmission following death of the host may be through either the hypnospore or infective zoospores (Villalba *et al.*, 2004).

Isolation of hypnospores from decaying mollusc tissue or from tissue culture in RFTM results in the production of zoospores when the hypnospores are placed in seawater. In vitro zoosporulation is mediated by both temperature and salinity with threshold values below which zoosporulation does not occur (Chu and Greene, 1989; Auzoux-Bordenave *et al.*, 1995; Ahn and Kim, 2001; Casas *et al.*, 2002). Auzoux-Bordenave *et al.* (1995) achieved sporulation of *P. olseni / P. atlanticus* at temperatures of 24° and 28° but not at 7° and 15°. Ahn and Kim (2001) found increased developmental rates and zoospore production with increasing temperature and achieved maximum rates of sporulation at temperatures greater than 20° but with some production of zoospores occurring at temperatures as low as 5°. Casas *et al.* (2002) found the optimum

temperature range for zoosporulation was 19 – 28°C with lesser production of zoospores down to 15°. Zoospores held at low temperatures remained viable for several months and gave rise to viable zoospores after they were transferred to higher temperatures (Ahn and Kim, 2001; Casas *et al.*, 2002). It has been postulated that the hypnospore may be a dormant stage in the life-cycle of *Perkinsus* spp. given that hypnospores can survive for extended periods under conditions that prevent zoosporulation without losing ability to zoosporulate (Chu and Greene 1989; Casas *et al.* 2002).

Extension of the geographic range of *Perkinsus* spp. infections may be mediated through passive or active mechanisms of transmission. Many organisms have been introduced into new regions through translocation by human activities such as shipping and aquaculture (Cohen and Carlton 1998; Galil, 2000). Any human activity that involves movement of infected hosts, including commercial or recreational fishing of host species, potentially facilitates transmission of the parasite and extension of range. Predators of hosts may also be active vectors of the parasite. Hoese (1967) found that *Perkinsus* trophozoites survived passage through the intestines of fish and Goggin *et al.* (1990) observed motile zoospores in faeces recovered from fish that had been fed tissue infected with *Perkinsus* sp. Passive transmission of the parasite from host to host in close proximity to one another and transport of *Perkinsus* cells (trophozoites, hypnospores or zoospores) assisted by ocean currents represent passive means of transmission.

Factors affecting spatial and temporal variations in prevalence and intensity of perkinsosis

Spatial and temporal variations in both the prevalence and intensity of *Perkinsus* spp. infections are affected by multiple factors that affect the parasite, the host or their interaction (Villalba *et al.*, 2004). The greatest research effort has focused on *P. marinus* infecting oysters in the U.S.A. and the effects of temperature and salinity on annual cycles of prevalence and infection intensity and geographic range.

Perkinsus prevalence and infection intensity are positively correlated with salinity. *P. marinus* has colonised low-salinity areas that have undergone elevated salinity following droughts, and once established in these areas, persists following decreases in salinity to normal levels. When salinity increases again, so does the prevalence and intensity of *P. marinus* infections (e.g., Villalba *et al.*, 2004).

The annual temperature cycle determines a seasonal pattern of variation in prevalence and intensity of *Perkinsus* spp. infections in hosts. Prevalence and intensity of *P. marinus* infections in oysters begins in Chesapeake Bay in spring when water temperature increases above 20° and reach a maximum in late summer. Mortality of oysters is concentrated in late summer and this is also the time at which new infections are mainly acquired. It is important to note, however, that transmission of *P. marinus* can also occur when host mortality is low or absent (Ragone Calvo *et al.*, 2003b). If water temperatures during spring and autumn are unusually high, this provides an extended period for parasite proliferation and results in greater mortality. Villalba *et al.* (2004) conclude that water temperature is probably the most important environmental factor affecting large-scale geographic distribution. Co-incident with a pronounced warming of water temperature in 1990-91, a 500 km extension of the range of *P. marinus* (to higher latitudes on the east coast of the U.S.A.) occurred. Patterns of infection and mortality were similar to the more southern locations where the parasite had been enzootic for decades (Ford, 1996; Ford and Smolowitz, 2007). In vivo and in vitro laboratory studies and field studies demonstrate the importance of both temperature and salinity on the activity and viability of *P. marinus* trophozoites, prezoosporangia and zoospores. In vivo experiments have also demonstrated the influence of both temperature and salinity on infectivity and disease progression (Villalba *et al.*, 2004). In particular, experiments to investigate the synergistic effects of temperature, salinity and infective cell concentration concluded that temperature was the most important factor, followed by infective cell dose and then by salinity in determining to susceptibility of oysters to *P. marinus* (Chu and Volety, 1997).

Susceptibility to infection and resulting disease intensity was positively related to water temperature, within the 10 – 25° range studied (Chu and La Peyre, 1993a, 1993b).

Epizootiological knowledge for other species of *Perkinsus* is limited compared to *P. marinus* but available studies support the importance of water temperature in determining the dynamics of *Perkinsus* infections. A 5-year survey incorporating monthly sampling of *P. olseni / P. atlanticus* infections in the clam *T. decussates* in Spain showed seasonal fluctuations in prevalence and intensity of infections whereby they peaked in spring/summer/autumn (maximal temperatures 19 – 21°) and regressed in late autumn/winter/early spring (minimal temperatures 9 – 10°). The greatest mortality of clams occurred in early autumn (Villalba *et al.*, 2005). The importance of temperature and salinity to cell activity and the viability of zoosporulation stages of *P. olseni / P. atlanticus* has been confirmed in vitro (Auzoux-Bordenave *et al.*, 1995; Ahn and Kim, 2001; Casas *et al.*, 2002).

Less studied than temperature and salinity, other stressors such as pollution (Chu and Hale, 1994), hypoxia (Sobral and Widdows, 1997; Cheng *et al.*, 2004) and decreased food availability (Powell *et al.*, 1996) may increase host susceptibility to infection. Indeed, a recent review of stress and immune responses in abalone (Hooper *et al.*, 2007) notes the link between increased stress and decreased immune functional capacity.

Population density of hosts also affects disease transmission. Greater rates of transmission would be expected in dense rather than sparse populations. Further, as the distance between infected and infection-free individuals increases, so does the dilution of *Perkinsus* cells. The importance of dosage of infective cells has been demonstrated for *P. marinus* (Chu and La Peyre, 1993b; Chu and Volety, 1997).

Several studies have found greater prevalence of *Perkinsus* infections in adult compared to juvenile hosts (e.g., Burreson and Ragone Calvo, 1996; Soniat, 1996) and similarly, positive correlation between infection intensity and age of host (Villalba *et al.*, 2005). Greater filtration rates and longer exposure to infective cells may explain these observations (Villalba *et al.*, 2004).

6.1.2. Epizootiology of P. olseni and Perkinsus sp. *in mollusc hosts in Australia*

Temperature-dependence

The importance of temperature as a factor affecting prevalence of *P. olseni* infections in abalone, infection intensity and mortality of abalone is also demonstrated by previous studies done in Australia.

During the research from which *P. olseni* was originally described (Lester and Davis, 1981), 10 blacklip abalone, infected with *P. olseni*, collected from a site near Port Lincoln in South Australia were kept in recirculating sea-water in laboratory tanks, 5 at 15° and 5 at 20°. Smears of the contents of lesions from the surface or within the musculature of the foot revealed both live and dead parasites. Live trophozoites and schizonts were commonly encountered in the pustules and haemolymph of abalone kept at 20°C. Dead trophozoites and schizonts were predominant in abalone kept at 15°C. Based on these observations, it was hypothesized that the balance between host and parasite was temperature dependent.

Two small-scale laboratory experiments concerning temperature-dependent mortality in blacklip abalone were described by Lester and Hayward (2005). Blacklip abalone, sourced from a South Australian hatchery, were exposed to *P. olseni* zoospores and then maintained in 15° seawater for up to 5 months resulted in no mortality. In a 2nd experiment, juvenile blacklip abalone, obtained from the NSW Brackish water research Station (Port Stephens, NSW) were exposed to *P. olseni*

zoospores then maintained at 19° for 6 months. There was no significant mortality relative to controls.

Temperature dependent mortality of greenlip abalone was examined in the laboratory by Goggin and reported in Goggin and Lester (1995). Greenlip abalone were infected with *P. olseni* and maintained in seawater at 15°C and 20°C.High mortality, relative to uninfected controls, occurred at both temperatures. There were greater numbers of parasites in abalone kept at the higher temperature. Temperature-dependent mortality occurred for the cockle, *A. trapezia*, with no difference in mortality between control cockles and those infected with *P. olseni* in water of 20°C but significantly greater mortality of infected cockles maintained at 27 – 30°C compared to controls (Goggin and Lester, 1995).

In summary, hypotheses concerning the temperature-dependence of mortalities due to *P. olseni* in wild populations of blacklip and greenlip abalone in South Australia and the limited laboratory experiments described for both abalone species and the cockle *A. trapezia* suggest a relationship between water temperature and infection intensity and subsequent mortality.

Host-specificity

Perkinsus infections have been found in many species at multiple locations around Australia and, in particular, 9 species including blacklip and greenlip abalone around the Yorke Peninsula in South Australia (Goggin and Lester, 1995). Moreover, *Perkinsus* isolated from 8 mollusc species were shown to have low host specificity in the laboratory (Goggin *et al.*, 1989). Despite, the apparent low host-specificity of *Perkinsus* in these instances, Lester and Hayward (2005) found no infections in 36 mollusc taxa other than blacklip abalone and one other species of abalone (*Haliotis roei*), at Taylor and Grindal islands, South Australia, although density of other species was sparse (B. Lester, pers. comm.).

In the only survey of alternative mollusc hosts for *Perkinsus* sp. in NSW, Gill (2003) detected no infections among the 190 specimens sampled at Wanda Wanda Head near Port Stephens, 7 specimens sampled from "Drum and Drumsticks" at North Jervis Bay or the 152 specimens sampled from St Georges Basin, Sussex Inlet and Burrill Lake in 2003. Species sampled by Gill (2003) at these locations included: *Pinna bicolour*, *Scaeochlamys* sp., *A. trapezia*, *Trichimya* sp., *Haliotis coccoradiata*, *Donnax deltoides*, *Turbo torquatus* and *Australium* spp.

6.1.3. Hypotheses regarding the epizootiology of perkinsosis and Perkinsus-related mortality of blacklip abalone in NSW

Initially, it is important to distinguish between the epizootiology of perkinsosis (i.e., *Perkinsus* infections in abalone) and the epizootiology associated with the mass mortalities of abalone that have been observed since the early 1990s in NSW. One of the important objectives of this chapter is to assess the likelihood of the involvement of *Perkinsus* sp. in these mass mortalities compared to alternative hypotheses that do not involve *Perkinsus* sp. If, for example, it is concluded that some factor other than *Perkinsus* sp. was the cause of observed mortality events then there would be a need to understand both the epizootiology associated with the causative factor as well as the epizootiology associated with *Perkinsus* sp. (that clearly causes disease, see Chapter 5), but under this scenario, not mortality. Alternatively, if it is concluded that *Perkinsus* sp. is the single factor or one of multiple factors that together are responsible for the observed mortality events, then there is only the need to consider the epizootiology associated with the combination of factors that together are causative.

Interpreting results from the NSW component of the NSDA, Callinan and Landos (2006) noted that the pattern of mortality among abalone in NSW was not one that has been matched in other abalone populations where *P. olseni* was known to be present, either in the extent of the stock

decline or the nature of the lesions. They suggested that either the NSW abalone were more susceptible, perhaps a more stressed population, or that the pathogen was a more virulent strain, or that other factors increased the natural susceptibility of abalone to this disease in NSW. Callinan and Landos (2006) speculated about alternative hypotheses regarding the epizootiology of perkinsosis and the subsequent mortality of abalone in NSW. One such hypothesis was that the pathogen had been present in abalone or other mollusc populations for a long time, along the entire NSW coast, and that since 1992, conditions had favoured disease outbreaks. However, they noted that the prevalence of *Perkinsus* sp. infections was greatest at the margins of the infected zone of coast, that this observation was consistent with a propagating epizootic entering a naïve population of abalone.

Adopting an epidemiological definition of the cause of a disease as *An event, condition or characteristic that plays an essential role in producing an occurrence of the disease* in an individual, we use the terminology of "component" and "necessary" factors within a "sufficient cause" complex (e.g., Thrushfield, 2007). A sufficient cause (or more accurately, "sufficient cause complex") is a set of minimal conditions that inevitably produces disease in an individual. A sufficient cause comprises one to many component factors. A necessary factor is a component of every sufficient cause. There may be a range of possible sufficient causes (i.e., sufficient cause complexes).

A range of hypotheses regarding alternative component factors and sufficient causes for the observed mass mortalities of abalone along the NSW coast are considered here:

Epi-Hyp-1: A pathogen (other than *Perkinsus* sp.) is necessary and sufficient

Epi-Hyp-2: Water temperature (above some threshold) is necessary and sufficient

Epi-Hyp-3: An environmental factor other than water temperature (above some threshold) is necessary and sufficient

Epi-Hyp-4: *Perkinsus* sp. is a necessary factor

Epi-Hyp-4.1: *Perkinsus* sp. is sufficient

Epi-Hyp-4.2: A pathogen (other than *Perkinsus* sp.) is a component factor

Epi-Hyp-4.3: An environmental factor other than water temperature (above some threshold) is a component factor
These component factors are sufficient

Epi-Hyp-4.4: Water temperature (above some threshold) is a component factor

Epi-Hyp-4.4.1: *Perkinsus* sp. and water temperature are sufficient

Epi-Hyp-4.4.2: Other factor(s) present in sufficient cause complex

Associated with the hypotheses that involve *Perkinsus* sp. as a necessary factor and an environmental factor as an additional component factor (Epi-Hyps: 4.3, 4.4, 4.4.1, 4.4.2), there are 2 additional hypotheses to be considered regarding the historical distribution and spread of *Perkinsus* sp. on the NSW coast:

Hist-Distribn-Hyp-1 *Perkinsus* sp. has been endemic along the coast of NSW since many years prior to the documented mass mortalities and identification of *Perkinsus* sp. in abalone;
(and that spatial and temporal variations in an environmental factor determines the spatial and temporal distribution of detectable *Perkinsus* sp., pathogenicity and subsequent mortalities).

Hist-Distribn-Hyp-2 *Perkinsus* sp. was not endemic to the entire coast of NSW for many years prior to the documented mass mortalities and identification of *Perkinsus* sp. in abalone;
(and that the presence of *Perkinsus* sp. infections in abalone at a location at a point in time is closely associated with the arrival of *Perkinsus* sp. at that location).

The implication of "Hist-Distribn-Hyp-1" is that the environmental factor (water temperature or other) must be identified for which spatial and temporal variations in that variable are closely related to the spatial and temporal patterns of detectable *Perkinsus* sp. and mortalities. Under "Hist-Distribn-Hyp-2", it is the combination of the spread (or introduction) of *Perkinsus* sp. to a location, combined with the level of the environmental variable (e.g., water temperature above some threshold level) that determines whether *Perkinsus* sp. is: detectable but infections are sub-lethal; or detectable and infections develop to intensities that cause mortality.

Due to retrospective nature of this study and absence of base-line data about the presence of *Perkinsus* sp. prior to observed mortality events, none of the possible causes for mass mortalities of abalone can be ruled out. In considering the hypotheses of cause it is, however, useful to assign to each one a categorical likelihood ("low", "medium" or "high") of being correct, to reflect the consistency of available data with the hypotheses. Obviously, future research outcomes may inform a revision of the likelihood assigned to these hypotheses.

To evaluate the relative likelihood of these alternative hypotheses, information presented in previous chapters is supplemented in this chapter with data and analyses that further inform evaluation of the epizootiolgy of perkinsosis and the observed mortality events. In addition, several other aspects of epizootiology are considered. A survey to identify alternative mollusc hosts for *Perkinsus* sp. informs understanding of the role of reservoir hosts in the spatial and temporal dynamics of perkinsosis. Analyses to identify differences in the prevalence and intensity of *Perkinsus* sp. infections between genders and among sizes of abalone inform an understanding of impacts of perkinsosis within the abalone population. Vectors and mechanisms by which *Perkinsus* sp. could be translocated between locations including movements related to commercial and recreational fishing are considered. The possibility of translocating *Perkinsus* sp. by reseeding reefs with hatchery-bred juveniles, derived from brood-stock collected from locations on the NSW coast at which *Perkinsus* sp. was known to be present is also evaluated.

Thus, the principal objectives of this component of the project, presented in this chapter, were to:

(i) Examine the effect of abalone gender on the prevalence and intensity of *Perkinsus* sp. infections;

(ii) Examine the effect of abalone size on the prevalence and intensity of *Perkinsus* sp. infections;

(iii) Implement a survey to monitor abalone abundance and mortality adjacent to the southern "front" of infection at South Jervis Bay;

(iv) Test for the presence of *Perkinsus* sp. in other potential mollusc hosts at locations where the parasite was present in abalone.

(v) Examine the potential for translocation of *Perkinsus* sp. during fishing and related activities;

(vi) Examine the potential for translocation of *Perkinsus* sp. during reseeding and enhancement experiments;

(vii) Identify environmental factors that were noted by divers to be associated with mortality events and changes in abundance of abalone;

(viii) Identify associations between water temperature, observed mortality events and infection intensity;

(ix) Investigate the availability of data about water quality and therefore, the potential to examine associations between water quality and observed mortality events.

(x) Consider the relative likelihood of alternative hypotheses about the epizootiology of perkinsosis and *Perkinsus*-related mortalities of blacklip abalone in NSW.

6.2. Methods

6.2.1. Effect of abalone gender on prevalence and intensity of Perkinsus sp. infections

The gender of each abalone sampled during the broad-scale survey was recorded as male, female or indeterminate. Chi-squared and Fisher's Exact tests were used to identify significant differences in the prevalence of *Perkinsus* sp. infections in male and female abalone in the pooled data-set and the samples from each survey location. Fisher's Exact tests were used to test for significant differences in the intensity of *Perkinsus* sp. infections (grades of infection from Ray's test on gill tissue) in male and female abalone.

6.2.2. Effect of abalone size on prevalence and intensity of Perkinsus sp. infections

All abalone sampled during the 2005 broad-scale survey were measured and assigned to one of 4 length-classes ($\leq$ 80 mm, 81 – 100 mm, 101 – 120 mm, and > 120 mm). Chi-squared and Fisher's Exact tests were used to identify significant differences in the prevalence of *Perkinsus* sp. infections in abalone among the 4 size-classes. The same tests were used to identify significant differences in the intensity of *Perkinsus* sp. infections among size-classes. For both prevalence and intensity, data was analysed for all locations pooled and at the level of individual locations.

6.2.3. Survey to monitor abalone abundance and mortality adjacent to the southern "front" of Perkinsus sp. infections at South Jervis Bay

The site "St George's Head South" was the most southern site within location "South Jervis Bay" at which *Perkinsus* sp. was detected during the 2005 broad-scale survey. Approximately 0.5 km to the west of this site at "St. George's Head West", 8 fixed sites were identified at which abalone were abundant (i.e., greater abundance than other sites at South Jervis Bay). These fixed sites (or crevices) were mapped and abalone within each fixed site were counted (using the same methods as described for the fishery-independent survey of abalone abundance, see Section 3.2.3). At each fixed site, two replicate counts, by different divers, were made of all abalone in three length classes, small (< 60 mm), medium ($\geq$ 60 mm and < 115 mm) and large ($\geq$ 115 mm). The initial

survey of these fixed sites occurred in April 2005. These sites were subsequently surveyed again in March 2006 and October 2006.

Ongoing monitoring of the abundance of abalone at these fixed sites is essentially a surveillance program for any extension of the range of *Perkinsus*-related mortality within this section of the NSW coast. The objectives of repeat surveys of these sites were: (i) to detect any sudden decreases in abundance of abalone; (ii) note the presence of any moribund or dead abalone and potentially (iii) sample moribund and dead abalone for post-mortem examination (macroscopic and histology).

6.2.4. Survey to identify alternative mollusc hosts for Perkinsus sp.

To identify alternative mollusc hosts for *Perkinsus* sp., other molluscs that were found close to abalone were collected from 2 sites within location North Jervis Bay (Beecroft Head and Targets) and the site St Georges Head within location South Jervis Bay. These sites were selected because *Perkinsus* sp. infections were prevalent at these sites when sampled during the broad-scale survey. The logic being that we would be most likely to detect *Perkinsus* sp. infections in other species at sites where *Perkinsus* sp. infections were relatively prevalent in abalone. Four species of mollusc were collected from the three sites: *Cabestana spengleri* (n = 42); *Thais orbita* (n = 79); *Granata imbricata* (n = 25); and *Trichomya hirsuta* (n = 30). For any given species found at a site, a minimum of 20 individuals were collected from the site. All samples were kept alive and stored within eskies.

Processing of these samples occurred within 24 hr of collection. For Ray's test only, a partial cross-section of tissue (~ 2 cm) including gill, mantle, gut, gonad (occasionally) and muscle (for *G. imbricata*) was excised from each individual (using sterile instruments) and placed into sterile tubes containing 20 ml of RFTM fortified with antibiotics. The diagnostic method for Ray's test was as previously described for abalone (Section 4.2.1.3).

6.2.5. Potential for transmission of Perkinsus sp. by translocation of abalone during fishing and related activities

To identify fishing practices that may have contributed to the transmission of *Perkinsus* sp. within and between sub-zones of the fishery, commercial abalone divers were asked a series of questions relating to their movements, practices and the movement of abalone within and between areas (Appendix 3 in Section 12.3). The specific questions were:

(Question 7)

What was the typical distance you would cover in a day's fishing? What was the minimum and maximum distance you would travel in one fishing day?

(Question 15a)

What fishing methods did you use (include how abalone were transported on the boat) and did you employ a deckhand? If there was a change in fishing methods please specify the year(s) of change and provide details.

(Question 15b)

What method did you use to measure legal size abalone (in water or on the boat)? Also what did you do with the under-size abalone?

(Question 15c)

Did you hang your abalone? If so please provide details (when and where; weight of abalone hung).

We also considered changes in regulations governing the fishery that may have affected fishing practices and the possibility of transmission of the parasite among areas (e.g., shucking at sea, closures).

6.2.6. Potential for transmission of Perkinsus sp. through translocation of abalone during reseeding and enhancement experiments

Between 1999 and 2001, experiments concerning the potential for enhancement of wild abalone populations using hatchery produced seed were based from the NSW Fisheries (and subsequently, NSW DPI) aquaculture/hatchery facility located at Tomaree, Port Stephens (Heasman *et al.*, 2004). Experiments involved the collection of brood-stock from wild populations of blacklip abalone at various sites along the coast of NSW and the subsequent release of hatchery-reared larvae and juveniles at various sites along the coast of NSW.

We examined the spatial and temporal pattern of (i) the collection of brood-stock and (ii) the subsequent release of larvae and juveniles, to assess the possibility that the *Perkinsus* sp. parasite may also have been translocated among locations on the NSW coast during these experiments.

6.2.7. Environmental factors identified by divers that were associated with mortality events and changes in abundance of abalone

During structured interviews of commercial and recreational divers, several questions related to changes in environmental variables that divers may have observed, specifically:

Commercial divers (Questions 12b and 12c):

12 (b). For each of years fished did you notice large changes in habitat, ocean temperature, water quality or other environmental factors?

12 (c). Can you recall exceptional environmental events (floods, storms, sand inundation) that lead to either an increase or decrease in abundance of abalone in an area? Describe your observations [This question was to get at cause of sudden decline, within year]

Recreational divers

7. Specific years when relative abundance of abalone had either noticeably increased or decreased. Prior to this change did you notice major changes in habitat, ocean temperature, water quality or other environmental factors?

6.2.8. *Associations between water temperature, observed mortality events and infection intensity*

Sea surface temperature (SST) data was obtained from 2 separate sources, satellite SST obtained from NASA and SST recorded by Waverider buoys located offshore from Sydney and Bateman's Bay (owned by NSW Department of Natural Resources; data collected and provided by Manly Hydraulics Laboratory, NSW Department of Commerce).

Average monthly SST data for selected areas along NSW coast for years 1985 to 2006 were downloaded from NASA satellite information via the PO.DAAC Ocean ESIP Tool (POET). The NSW coast between Diamond Head in the north and Wonboyn in the far south was divided into eight geographic areas (locations), each between 20 – 30 nautical miles latitude (Table 6.1). For each area, the eastern longitude was defined as the edge of the Continental Shelf (~200 m depth) and the western longitude as the shoreline. The sea surface temperature data were downloaded from two satellites – for the period 1985 to 2004 and 2006 AVHRR Pathfinder V5, and for 2005 MODIS/ Aqua. For each year, SST data was extracted with the following specifications: daytime readings; spatial resolution of 4 km; and "high quality" readings (i.e., minimum quality = 4, the default for AVHRR Pathfinder or "best" for MODIS/ Aqua). The algorithm specified for MODIS/ Aqua was Far-IR.

Table 6.1. Geographic areas for SST data.

Location	Geographic range	Range of latitude (nm)	Latitude		Longitude	
			N	S	W	E
North Coast	Diamond Hd to Forster	30	-31°40'	-32°10'	152°40'	153°05'
Port Stephens	North of Broughton Is to Stockton	20	-32°30'	-32°50'	152°10'	152°42'
Central Coast	Red Head to Cape Three Points	30	-33°00'	-33°30'	151°34'	152°10'
Sydney	Pittwater to Port Hacking	30	-33°35'	-34°05'	151°15'	151°45'
Kiama	Bulli to Black Head	27	-34°20'	-34°47'	150°52'	151°10'
Jervis Bay	Crookhaven to Bendalong	20	-34°55'	-35°15'	150°47'	151°00'
South Coast	Batemans Bay to Narooma	30	-35°43'	-36°13'	150°10'	150°30'
Far South Coast	Tathra to Wonboyn	30	-36°45'	-37°15'	149°57'	150°18'

Satellite SST data were based on averages from 4 km square grids and spanned the area between the coast and edge of the continental shelf. It was important to determine whether these data (available for all locations off the NSW coast) provided indices of water temperature that were consistent with indices from the Waverider buoys that were located at only 2 locations: 12 km offshore of Sydney and 8 km offshore of Bateman's Bay (South Coast). Monthly SSTs from each source were plotted against one another for each of the 2 common locations, Sydney and Bateman's Bay (South Coast) and correlations were calculated.

For each location, using satellite SST data, the following indices were calculated and plotted: mean SST for the period 1985 – 2006 (i.e., the grand mean); mean quarterly (January – March, April – June, July – August, October – December) SST, the one-year moving average of SST (i.e., previous 4 quarters); mean October – June SST (i.e., excluding July – September, the quarter when water temperature was coldest). Associations between these indices of water temperature and (i) timings of observed mass mortality events; and (ii) intensities of *Perkinsus* sp. infections in 2002 and 2005 were then examined. The consistency of such associations with alternative hypotheses concerning the epizootiology of perkinsosis and *Perkinsus*-related mortalities (Section 6.1.3) could then be evaluated. The rationale for considering the range of indices of water temperature was that

other studies have demonstrated that infection intensity, transmission and host-mortality for *Perkinsus* spp. in other mollusc hosts are a function of water temperature and the duration of elevated water temperature (Section 6.1.1). Mean quarterly SST provides an index of absolute STT at the quarterly scale. One-year moving average SST (calculated quarterly) provides an index that, for any given quarter, represents the mean temperature during the previous 12 months (which may affect the opportunity for *Perkinsus* sp. infections to increase in prevalence and intensity develop). Mean October – June SST provides an index of water temperature corresponding to the 3 quarters (Autumn, Summer and Spring) within which rates of transmission, prevalence and intensity of Perkinsus spp. in other hosts are maximised.

6.2.9. Availability of data about water quality and the potential to examine associations between water quality and observed mortality events

The availability of data concerning water quality, the spatial and temporal scale of monitoring and the parameters of water-quality measured were assessed through internet searches and enquiries with relevant NSW Government departments and organisations.

Comparison of (i) the spatial and temporal scales of available time-series of water-quality data with (ii) the spatial and temporal scale of observed mass mortalities of abalone and detection of *Perkinsus* sp. infections in abalone, was made to assess the potential for examining associations between water quality and observed mortality events.

6.3. Results

6.3.1. Effect of abalone gender on prevalence and intensity of Perkinsus sp. infections

Perkinsus sp. infections were more prevalent in female than male abalone sampled during the 2005 broad-scale survey (Chi-squared = 9.130, *df* = 1, *P* = 0.003). Within locations at which *Perkinsus* sp. infections in abalone were detected, *Perkinsus* sp. was detected in 18 / 363 (5.0%) male abalone, 45 / 413 (10.9%) female abalone and 2 / 52 (3.8%) abalone in which gender was indeterminate. Within individual locations, the proportion of abalone infected with *Perkinsus* sp. was generally greater for females than males (Fig. 6.1) but these differences were not statistically significant (Fisher's Exact $P > 0.05$ in all cases).

There was no significant difference in the intensity of *Perkinsus* sp. infections in male and female abalone for the pooled set of data ($P = 0.279$, Fisher's Exact test) or for individual locations (Fisher's Exact tests, $P > 0.05$ in all instances).

The significantly greater prevalence of *Perkinsus* sp. infections in female, compared to male, abalone contrasts with the equivalent data from the survey done in 2002. Within locations at which *Perkinsus* sp. infections were detected in abalone in 2002, *Perkinsus* sp. was detected in 20 / 240 (8.3%) male and 24 / 239 (10.0%) female abalone, there being no significant difference between these frequencies (Chi-squared = 0.419, *df* = 1, *P* = 0.517).

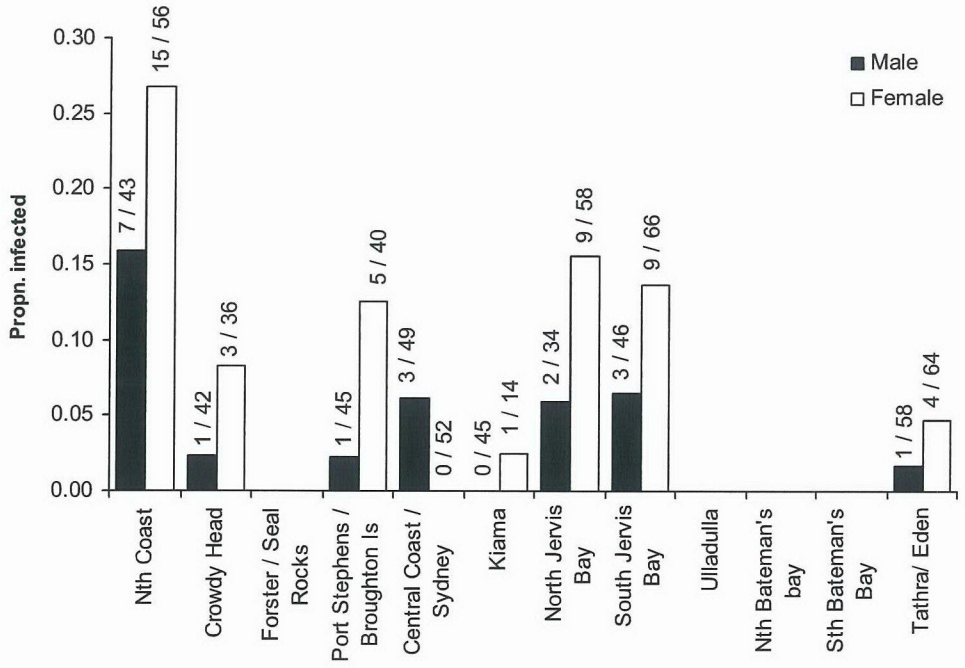


Figure 6.1. Prevalence of *Perkinsus* sp. infections in male and female abalone, by location.

6.3.2. Effect of abalone size on prevalence and intensity of Perkinsus sp. infections

There was no significant difference in the prevalence of *Perkinsus* sp. infections among the 4 size-classes of abalone sampled during 2005 (Fig. 6.2; Chi-squared = 0.317, $df = 3$, $P = 0.957$). Note that the pooled data-set excluded the locations "North Coast" and "Tathra – Eden" because no abalone < 80 mm were sampled from these locations. However, there were no significant differences identified among the 3 size-classes of abalone sampled from either of these locations (Fisher's Exact tests, $P = 0.764$ for "North Coast"; $P = 1$ for "Tathra-Eden"). Nor were there any significant differences identified among all 4 size-classes for the other individual locations (Fisher's Exact tests, $P > 0.05$ in all instances).

Intensity of infection did not differ significantly among the 4 size-classes of abalone, for either the pooled data-set ($P = 0.517$, Fisher's Exact test) or for individual locations ($P > 0.05$ in all instances, Fisher's Exact tests).

Results based on the 2005 broad-scale survey were consistent with those from 2002. Prevalence of *Perkinsus* sp. infections did not differ among 3 size-classes (≤ 100 mm, 101 – 120 mm, > 120 mm) of samples from the 2002 survey (Chi-squared = 0.0063, $df = 2$, $P = 0.997$).

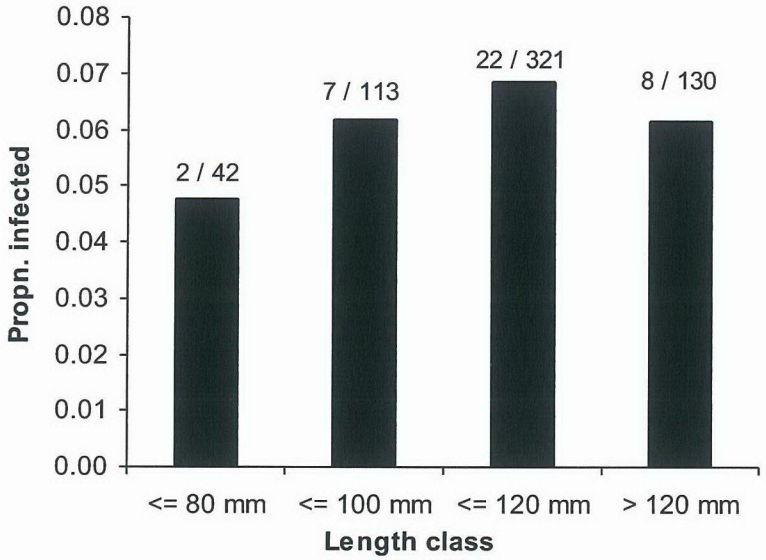


Figure 6.2. Prevalence of *Perkinsus* sp. infections in 4 size-classes of abalone. Note: data from locations "North coast" and "Tathra – Eden" excluded because no abalone < 80 mm sampled from these locations.

6.3.3. *Survey to monitor abalone abundance and mortality adjacent to the southern "front" of Perkinsus sp. infections at South Jervis Bay*

There was no significant decrease in abundance of abalone at 8 fixed sites to the west of St George's Head (Fig. 6.3). Consequently, there is no evidence of any significant mortality of abalone due to *Perkinsus* sp. or any factor, other than natural mortality, during the period April 2005 to October 2006. There were no observations of moribund or dead abalone at these fixed sites during March or October 2006.

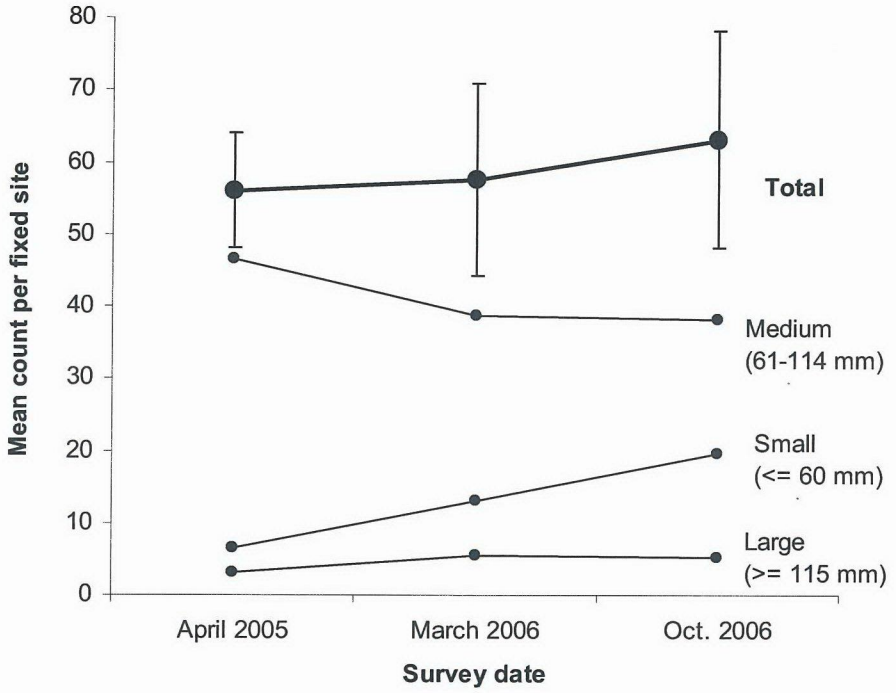


Figure 6.3. Mean abundance of abalone (small, medium and large size classes; and total +/- 1 SE) at 8 reference crevices at "St. Georges Head West" assessed during April 2005, March 2006 and October 2006.

6.3.4. *Identification of alternative mollusc hosts for Perkinsus sp.*

No *Perkinsus* sp. infections were detected (using Ray's test) in any of the four species of mollusc (total $n = 176$) sampled from 2 sites at North Jervis Bay (Beecroft Head and Targets) and the site at South Jervis Bay (St. Georges Head).

6.3.5. *Potential for transmission of Perkinsus sp. through translocation of abalone during fishing operations*

The closures to fishing for abalone (due to concerns about depletions of stock, see Section 3.1) in Zones F – L of Region 1 occurred between 1 and 4 years after observed mass mortalities of abalone and the detection of *Perkinsus* sp. within these zones. As effort by commercial abalone divers progressively transferred from zones G, H and J (Central Coast, Sydney, Wollongong) to zones F and K (Port Stephens and Kiama) during the mid 1990's and then to zone L (Jervis Bay) during 1999 – 2000 (Section 3.3.2), divers or activities related to harvesting and distribution of

product may have been acting as vectors for *Perkinsus* sp. through the translocation of diseased abalone or contaminated equipment. Activities that may result in translocation of *Perkinsus* sp. and operate at spatial scales between 10's of m and 1000's of km have been identified and are described here.

Harvesting at individual drops

When diving for abalone at individual drops (specific dive locations), divers accumulate their catch of abalone in mesh bags before returning their catch to their boat. At the completion of harvesting at a given drop, the fisher moves to another drop. Thus, during commercial or recreational diving at a specific drop, abalone are moved over distances of the order of 10's or 100's of metres from where they were collected.

Travelling between drops during a day's fishing

The mean "typical distance covered in a day's fishing" reported by divers during interviews was 22 km. The mean "maximum distance travelled in one fishing day" reported was 36 km. Distances reported for fishing in the vicinity of Jervis Bay were greater than for other areas, typically 40 km and a maximum of 60km. Thus, abalone harvested during the day's fishing were carried onboard vessels within and between sub-zones over distances of the order of 10's of km.

Following regulation preventing shucking of abalone at sea (in 1980), divers have used 2 methods to keep their catches of abalone alive during a fishing trip. The first method involves hanging of abalone over the side of the boat (especially in summer), the result being that abalone harvested from one area may be suspended in the water at another area or areas. The second method involves pumping sea-water over the catch in boxes on the deck of the boat with this water then being pumped or drained back into the ocean. With this method, water pumped over abalone caught from one area may be returned to the ocean in another area or areas.

Hanging of catch on multi-day trips

Hanging of catch was not restricted to maintaining the catch during single fishing days. On fishing trips up to several days duration, the catch from individual days of fishing was often "hung" at a holding location until the multi-day trip was completed and the accumulated catch transported to the processor. Weights of abalone that were hung ranged from 100's of kg up to 2 tonnes and for periods up to several days. Although interviewed divers reported that the frequency of "hanging" decreased through the 1990s, hanging of catch was still commonly used in the component of the fishery to the north of Port Stephens when fishing zone E. Thus, the spatial scale at which abalone, potentially infected with *Perkinsus* sp., were moved from where they were collected to where they were hung was/is of the order of 10's of km (up to approximately 100 km).

Movement of sub-legal abalone among reefs

Divers also reported during interviews that sub-legal abalone were often moved between reefs. This occurred when abalone harvested from one reef were subsequently measured on the boat and sub-legal abalone were subsequently returned to the water at a location other than that from which they were originally taken. Practices differed among divers and among years but this practice also resulted in the translocation of abalone, potentially infected with *Perkinsus* sp. The spatial scale at which this type of translocation occurred is also of the order of 10's of km.

Shucking of abalone and discard of viscera

Shucking of abalone at sea was banned in 1978. Moreover, with the majority of the commercial catch of abalone in NSW being live, the return of viscera to the water following shucking of

abalone is more associated with recreational fishers and illegal fishers. It is likely that shucking occurs relatively close to the locations at which abalone were harvested and thus, up to a scale of the order of 10's of km.

Movement of divers, boats, equipment between locations on the NSW coast

When commercial or recreational divers have moved from one location on the coast to another, across sub-zones, zones or regions, this has involved movement of boats and associated equipment and dive-gear across distances of the order of 10's or 100's of km. If this equipment was contaminated with viable cells of *Perkinsus* sp., then this would also represent a mechanism for the translocation of disease at the scale of 10's or 100's of km.

Movement of catches to processors and distributors

Similarly, movement of catches of abalone from the locations at which they were harvested to processors or distributors on the south coast of NSW (Ulladulla or Eden; Kiama during the 1990's) or Sydney represents another possibility for the translocation of disease to processors. Further translocation to ocean waters could then occur if processors discharged contaminated water from their holding tanks or disposed of abalone viscera into the ocean. This mechanism of translocation is at the scale of 100's of km or eventually 1000's of km when abalone are distributed to other states of Australia or internationally.

Distribution of abalone viscera as bait

Translocation of *Perkinsus* sp. via the distribution and sale of viscera to recreational anglers is another potential mechanism by which *Perkinsus* sp. may have been moved between areas. Abalone gut is a bait used by recreational anglers when rock fishing for several commonly targeted species, especially black drummer (*Girella elevata*). Whilst some anglers may have harvested abalone themselves and subsequently used the abalone gut as bait, the majority of anglers probably obtained the abalone gut from a retail outlet. Frozen abalone gut was widely sold at fishing shops and service stations along the coast of NSW until 2007 when a ban was implemented. Researching the detailed history of the locations from which abalone gut has been sourced and subsequently distributed was beyond the scope of this project. However, there were 3 companies known to have distributed abalone viscera into the NSW bait market: Twofold Bay Bait (based at Eden); Tweed Bait (based at Tweed Heads) and Bremer Bait (based at Clontarf, Queensland). They collectively sold about 7 t of viscera per annum into the NSW market with an estimated 1 t sourced from NSW processors and 6 t coming from Mallacoota in Victoria (J. Frances, I&I NSW, pers. comm.). The quantities and origins of abalone viscera supplied to the bait market historically are unknown. The distribution of abalone viscera does, however, represent another potential mechanism by which *Perkinsus* sp. may have been moved both locally and across large distances, at a scale of the order of 100's or 1000's of km.

6.3.6. *Potential for transmission of Perkinsus sp. during reseeding and enhancement experiments*

Blacklip abalone were collected from wild populations along the NSW coast and transported to the hatchery at Tomaree (Port Stephens) between 1999 and 2001. For experiments concerning spawning induction, abalone were collected from wild abalone populations at the following sites on the south coast of NSW:

- Eden in August and September 1999;
- Sydney in October 1999.

(Source: Table 2 in Chapter 4 in Heasman *et al.*, 2004).

Abalone were collected from the wild to provide brood-stock at the Tomaree facility between April 1999 and November 2000. These abalone were collected from sites at:

- Port Stephens and Broughton Island between April 1999 and September 2000;
- Eden in August 1999 and November 2000;
- Sydney in April 2000;
- Ulladulla in July 2000;
- Kiama in September 2000;

(Source: Table 3 in Chapter 4 in Heasman *et al.*, 2004).

There is no evidence that *Perkinsus* sp. was present in populations of abalone at Ulladulla or Eden when brood-stock were collected from these locations in 1999 or indeed, since that time (Chapter 4). However, mortalities of abalone had been reported and *Perkinsus* sp. had been identified in abalone at locations between the Central Coast and Kiama prior to the collections of abalone from Sydney in 1999 and 2000 and from Kiama in 2000. Although *Perkinsus* sp. was not identified in the abalone population at Port Stephens until the broad-scale survey in 2002, the documented "2000 mortality event" at Port Stephens based on observations of morbidity and mortalities during 1999 – 2001 (Chapter 3) suggests the presence of *Perkinsus* sp. at this time. It is therefore possible that *Perkinsus* sp. was transported into the hatchery at Tomaree from:

- Sydney in October 1999 or April 2000;
- Kiama in September 2000;
- Port Stephens during April 1999 – September 2000.

There is no definitive evidence regarding whether or not abalone introduced to the Tomaree hatchery collected from Port Stephens, Kiama or Sydney between October 1999 and November 2000 were infected with *Perkinsus* sp. No tests for the presence of *Perkinsus* sp. were done on abalone from the hatchery prior to 2002 (M. Heasman, pers. comm.). Two batches of juvenile abalone were tested for *Perkinsus* sp. in 2002-03 (M. Heasman, pers. comm.). A sample of 99 juveniles were tested using both Ray's test and histology as part of the NSW component of the NSDA and all tested negative for *Perkinsus* sp. (Callinan and Landos, 2006). Ray's test was performed on another sample of 150 juveniles supplied to Craig Hayward (University of Queensland) in July 2002. Of this sample of 150, tissue samples from 126 were placed in RFTM on the day they were received and subsequently all tested negative for *Perkinsus* sp. The remaining 24 abalone were held live in tanks for 27 days before tissue samples were removed, placed in RFTM and subsequently examined. Although 2 of these 24 samples tested positive for low-grade infection (Ray's grade 0.5), these results are confounded by the facts that: (i) recirculating seawater in the tanks in which these abalone were kept for 26 days was obtained from Moreton Bay (where *Perkinsus* sp. was present) and (ii) other molluscs which potentially had *Perkinsus* sp. infections were being maintained within the facility at the time (C. Hayward, pers. comm.). Following these equivocal results, no additional translocations were made of hatchery reared stock to other locations (M. Heasman, pers. comm.).

Because hatchery brood-stock was obtained from locations at which *Perkinsus* sp. infections in abalone were known to occur and there was no testing of samples of brood-stock or hatchery-reared juveniles prior to 2002, the possibility that hatchery-reared juveniles and larvae released at sites along the NSW coast cannot be excluded.

Juvenile abalone that had been spawned and reared at the hatchery and subsequently released into the wild comprised the release of:

- 446,000 juveniles at sites adjacent to Port Stephens between October 1998 and Feb. 2001
- 32,000 juveniles at 4 sites at Sydney in June 2001 and March 2002

- 36,000 juveniles at 4 sites at Kiama in December 2001
- 800 juveniles at Tura Pt., Merimbula in October 2001
- 344,000 juveniles at sites near Eden between November 1999 and September 2001

(Source: Table 2 in Chapter 8, Heasman *et al.*, 2004).

The range of sizes of juvenile abalone released was 1.3 –18 mm (shell length), but larger juveniles were released at 3 sites at Port Stephens in November 2000 (43 cm juveniles) and at Tura Pt. at Merimbula in October 2001 (40 cm juveniles).

Larval hatchery-reared abalone were also released into the wild at sites in Port Stephens, Sydney, south of Ulladulla (Brush Is.) and Eden, between January 1999 and February 2002 (Table 1 in Chapter 8, Heasman *et al.*, 2004).

If *Perkinsus* sp. infections were present in juveniles or larvae that were released into the wild, releases at Sydney and Kiama in 2001 and 2002 were within the area, Central Coast to Kiama, where *Perkinsus* sp. in wild abalone was already known to occur at a number of sites. However, releases at Tura Pt. (Merimbula) in October 2001 and at multiple sites in the vicinity of Eden and Disaster Bay, on the far south coast of NSW, between November 1999 and September 2001 were at locations at which *Perkinsus* sp. had not been detected at that time. *Perkinsus* sp. was first detected at Port Stephens in 2002 and may or may not have been present in wild abalone at Port Stephens prior to releases of larvae and juveniles from the hatchery between January 1999 and February 2002.

It is important to note here that there have been no reports of mass mortalities in the vicinity of Eden since the reseeding of abalone during 1999 – 2001 or at Merimbula since the reseeding in 2001. There is no evidence from the fishery-independent survey of abalone abundance that there have been significant declines in abundance of abalone consistent with such mortality events at these locations (Fig. 6.4). There is, however, the documented presence of *Perkinsus* sp. in abalone in 2005 at the Merimbula site, approximately 5 km south of Tura Pt, the site at which hatchery-reared juvenile abalone were released in October 2001.

6.3.7. Environmental factors identified by divers that were associated with mortality events and changes in abundance of abalone

During interviews, divers suggested several factors, including environmental factors, which may have contributed to declines in abalone abundances over the longer-term (illegal harvesting, habitat changes involving the invasion of *Caulerpa filiformis*). Divers also associated several factors with declines in abundance of abalone over shorter times scales (within a year), namely severe storms (encompassing floods, large seas, sand-up events), water quality (associated with sewage treatment plants); elevated water temperature; and intensive harvesting (see comments in Table 3.1; Section 3.4.1). Such observations suggest the importance of weather-driven mortality events and other factors (e.g., poor water quality) that may impact abalone stocks from time to time.

Notably, there were no environmental factors that were associated, by divers, with mortality events that were consistent across the mortality events identified in Section 3.3.1: the "2000 mortality event" at Port Stephens; the "1992 mortality event" on the Central Coast; the "1993 mortality event" at Sydney-Wollongong; the "1996 mortality event" at Kiama; and the 2000 – 2002 sudden decline in abundance at Jervis Bay.

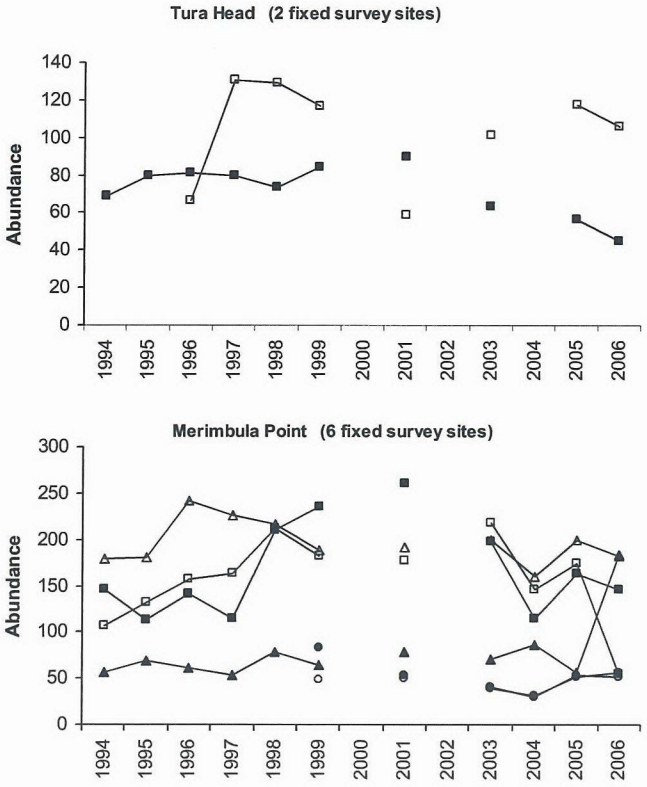


Figure 6.4. Estimated abundance of abalone from fishery-independent surveys of 2 sites at Tura Head and 6 sites at Merimbula Pt (1994 – 2006).

6.3.8. *Associations between water temperature, observed mortality events and infection intensity*

6.3.8.1. *Correspondence between satellite and buoy SST*

Monthly satellite SST and buoy SST were positively correlated ($R = 0.98$, $R^2 = 0.96$, $P < 0.01$) at Sydney and at Bateman's Bay ($R = 0.93$, $R^2 = 0.86$, $P < 0.01$) (Fig. 6.5). Satellite SST was greater than buoy SST by a mean 0.54°C at Sydney and 0.98°C at Bateman's Bay.

Based on the significant positive correlations between satellite and buoy SST, it was concluded that satellite SST provides an index of fluctuations in water temperature that occurs in water closer to the coast. Although satellite SST does not measure the absolute temperature of water immediately adjacent to the coast, where abalone occur, it is reasonable to assume that changes in water temperature in this zone will be indicated by changes in satellite SST. Satellite SST, available for all locations along the coast of NSW that were of interest in this project, could therefore be used as an index of quarterly and annual changes in inshore water temperature.

6.3.8.2. *Differences in water temperature among locations, seasons and years*

Patterns of mean seasonal (quarterly) water temperature were similar among locations with a mean intra-annual fluctuation in quarterly water temperature of 5.3°C (Fig. 6.6). Mean water temperature was lowest during the 3rd quarter of the year (July – September). Water temperature increased by a mean 2.0°C during the 4th quarter (October – December), then by a further 3.3°C during the 1st quarter (January – March) of the following year. Quarterly mean temperature then decreased from its maximum in the 1st quarter by a mean 2.6°C during the 2nd quarter (April – June) before decreasing by a mean 2.8°C during the 3rd quarter.

Mean annual water temperature decreased with increasing latitude (Figs. 6.6 and 6.7). Water temperature at the most southern location considered here, "Tathra – Eden" (18.5°C), was a mean 3.5°C lower than the most northern location, "North coast / Crowdy Head / Forster" (22.0°C).

There was, however, considerable variation in water temperature from year to year (Fig. 6.7). Generally, the pattern of annual water temperature between 1986 and 2006 was similar among locations. The magnitude of differences among years was, however, greater for the 4 southern locations than the 4 northern locations. The difference between the minimum and maximum mean annual temperature for the southern locations was 2.1 – 2.4°C compared to 1.3 – 1.5°C for the northern locations.

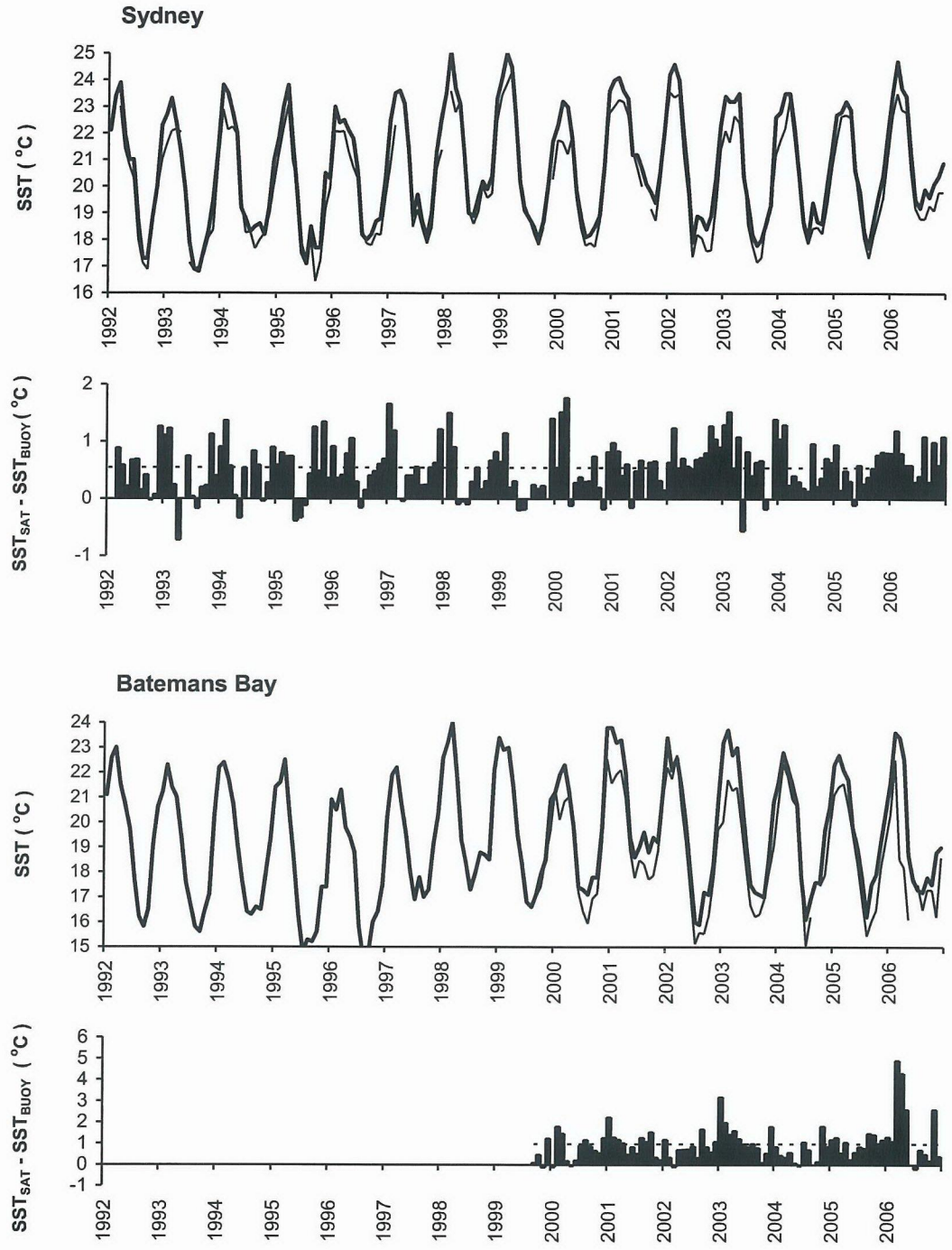


Figure 6.5. Association and difference between 2 sources of sea surface temperature, satellite SST (bold line) and buoy SST (fine line) at 2 locations, Sydney and Bateman's Bay. Horizontal dashed lines on graphs of differences indicate mean difference. SST data from both sources are monthly means.

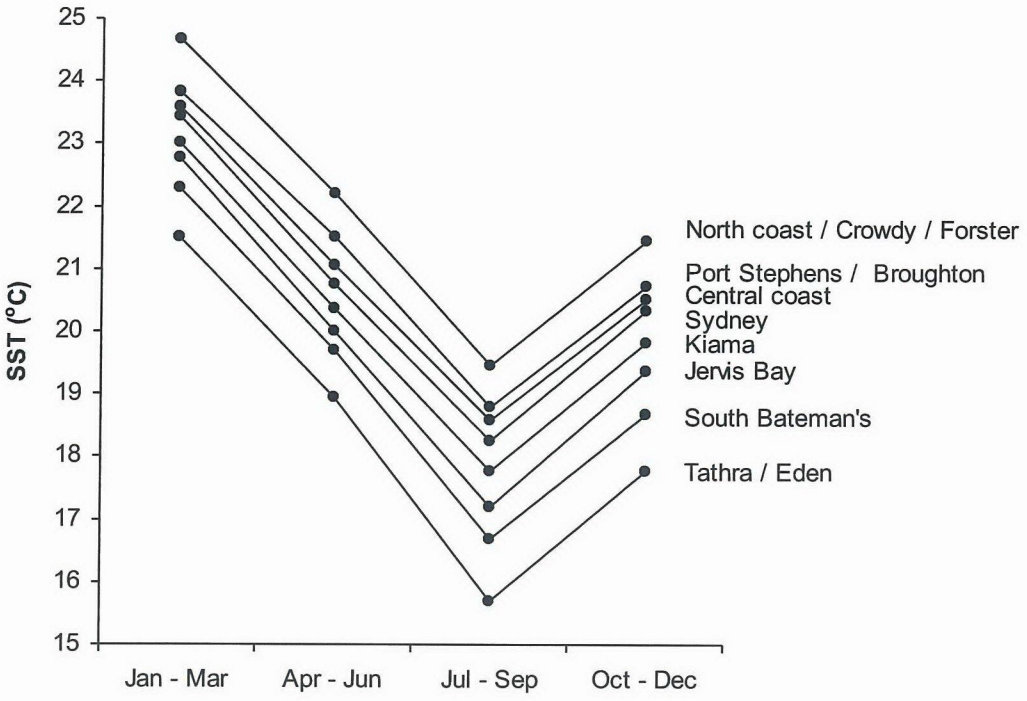


Figure 6.6. Mean seasonal (quarterly) satellite-SST at 8 locations along the NSW coast, between 1985 and 2006.

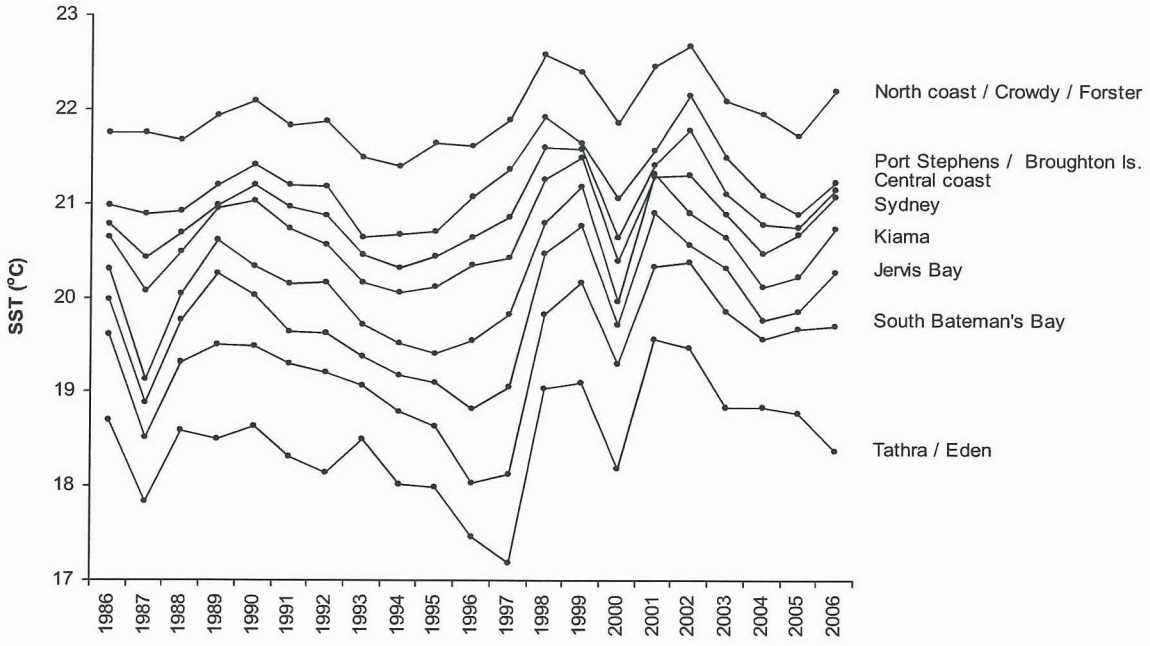


Figure 6.7. Mean annual satellite-SST at 8 locations along the NSW coast, between 1986 and 2006. Annual mean in each year calculated as the mean of temperatures during July – Dec of the previous year and January – June of the given year.

6.3.8.3. Associations between indices of water temperature and observed mass mortality events

The documented mortality events at specific locations in specific years (+/- 1 year) were not consistently associated with increases or decreases in any of the indices of water temperature examined (Fig. 6.8).

One-year moving average SST associated with mortality events: fluctuated within the range 21 – 22°C at Port Stephens, was relatively stable at approximately 21°C on the Central Coast, fluctuated within the range 20 – 21°C at Sydney, 19 – 20°C at Kiama and 20 – 22 °C at Jervis Bay. Compared to years prior to and since the mortality events, fluctuations in this index were not exceptional. It can, however, be stated that one-year moving average SST exceeded approximately 20°C during some part of the period associated with each mortality event at each location.

Fluctuations in mean October – June SST followed a similar pattern to one-year moving average SST, but at a greater temperature (because of the exclusion of July – September SST from this index) (Fig. 6.8). Mean October – June SST exceeded approximately 21°C during some part of the period associated with each mortality event at each location.

Similarly, there were no patterns in mean quarterly SST that were consistently associated with mortality events at each location. There was no consistent evidence across the mortality events of an association with exceptionally warm or cold water in any of the quarters. Maximum SST occurred in the first quarter (January – March) at each location and was 22 °C or greater during the years associated with mortality events.

In preparation for evaluation of the likelihood of alternative hypotheses regarding the cause of mortality events (in Section 6.4.1), it is important to note that indices of water temperature were greater than 22°C at "North Coast / Crowdy Head / Forster" in 2005, when low-intensity infections of *Perkinsus* sp. were found to be prevalent in abalone. In 2005, one-year moving average SST at "Tathra-Eden" was approximately 18.5°C and mean October – June SST was below 20°C.

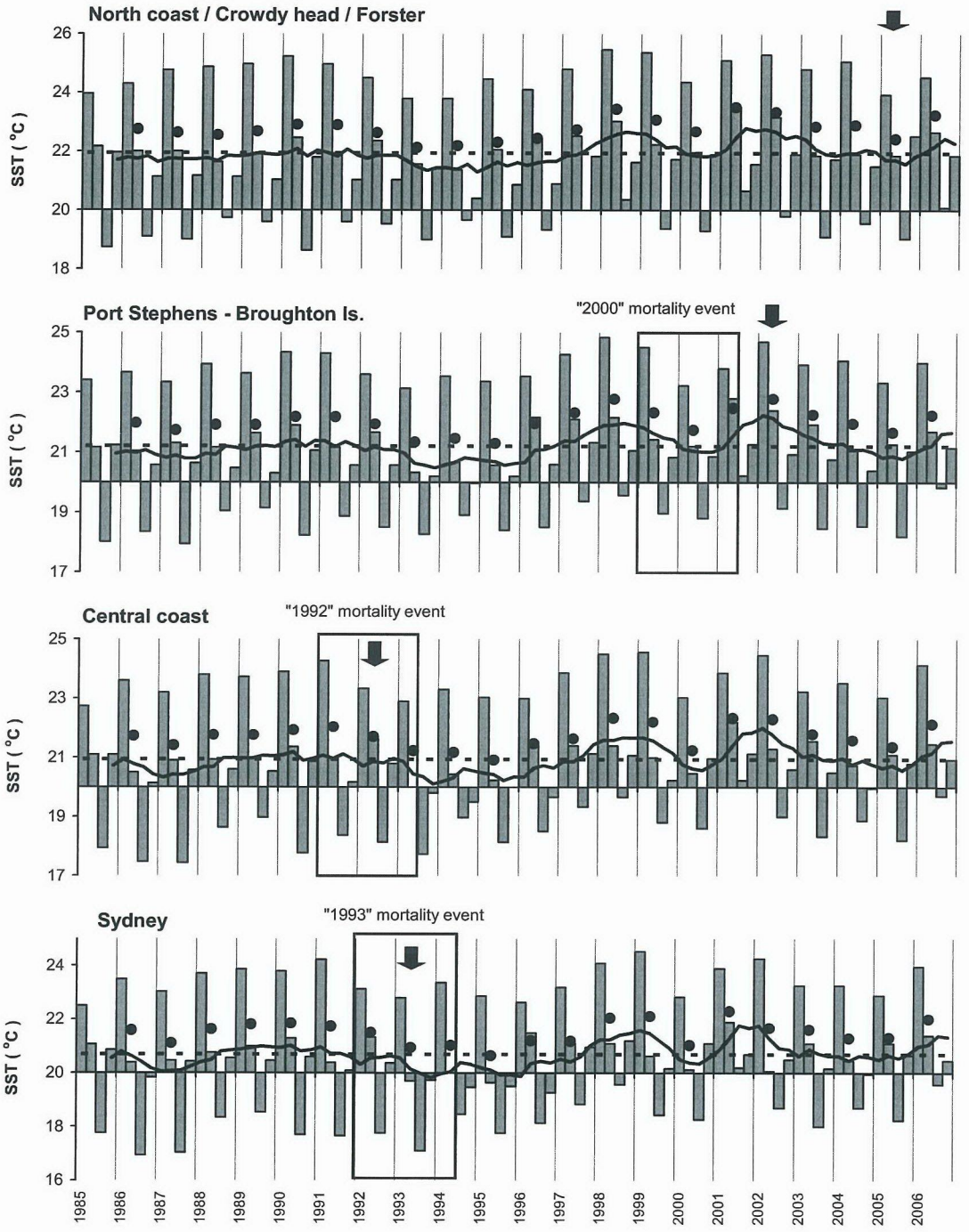
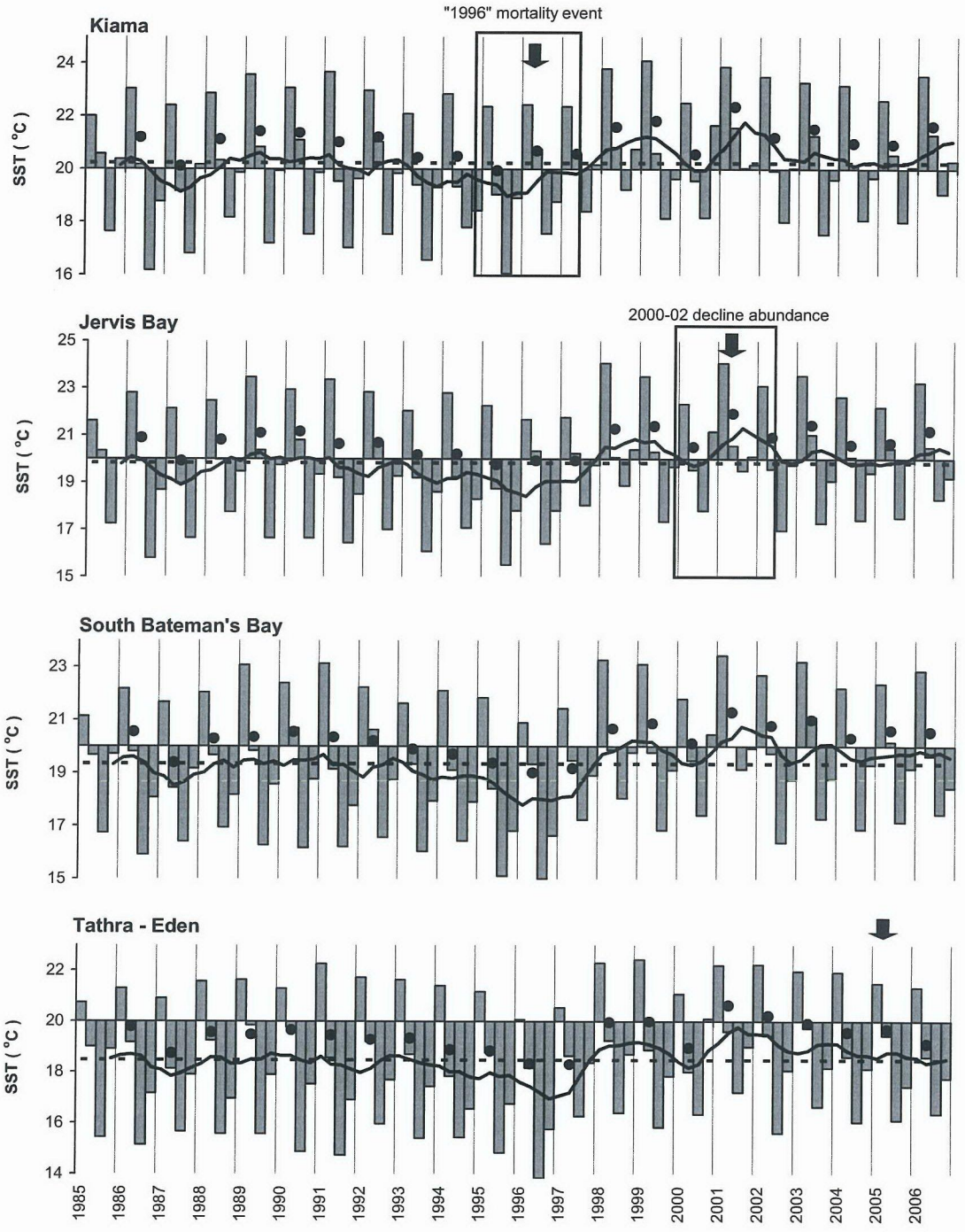


Figure 6.8. Relationship between observed mortality events and fluctuations in water temperature between 1985 and 2006 at 8 locations along the NSW coast.

Grey bars indicate quarterly mean satellite-SST; Horizontal dashed line indicates 1985 – 2006 mean SST; Bold line indicates moving 1-year mean SST; Black filled circles indicate mean Oct – Jun temperature (9 months; i.e., excluding Jul – Sep); Black arrows indicate first detection of *Perkinsus* sp. at each location.

Figure 6.8. (Cont'd)



Grey bars indicate quarterly mean satellite-SST; Horizontal dashed line indicates 1985 – 2006 mean SST; Bold line indicates moving 1-year mean SST; Black filled circles indicate mean Oct – Jun temperature (9 months; i.e., excluding Jul – Sep); Black arrows indicate first detection of *Perkinsus* sp. at each location.

6.3.8.4. *Associations between water temperature and intensity of Perkinsus sp. infection in 2002 and 2005*

Although there was no significant difference in the prevalence of *Perkinsus* sp. infections in abalone between 2002 and 2005, infection intensities were significantly greater in 2002. During 2002, 95% of infections were high-grade (Ray's grade ≥ 2) compared to only 31% from the 2005 survey (Section 4.3.5). It is noteworthy that water temperatures were lower in 2005, compared to 2002 by approximately 1°C (Fig. 6.7), the greater temperatures in 2002 being associated with greater infection intensity.

6.3.9. ***Availability of data about water quality and the potential to examine associations between water quality and observed mortality events***

The major time-series of data concerning water quality are summarised in Table 6.2 within 3 categories: location-specific monitoring, pre 1990's (Table 6.2.1); location-specific monitoring post-1990's (Table 6.2.2); beaches and waterways, faecal contamination (Table 6.2.3).

In general, these data are of limited use for the purpose of correlating parameters of water quality with the timing of abalone mortality events and the prevalence and intensity of *Perkinsus* sp. infections in abalone. In contrast to the data available about SST, the available time-series describing water quality do not cover the spatial and temporal scales at which abalone mortalities and detection of *Perkinsus* sp. have been documented. Time-series prior to the 1990's are limited to the Sydney region alone (Tables 6.2.1 and 6.2.3). Commencing in 1995, associated with the ocean outfalls of sewerage treatment plants, an expanded range of water-quality parameters were monitored by the NSW Department of Environment and Conservation. However, this monitoring has been limited to the section of coast between Sydney and Kiama (Table 6.2.2). Similarly, routine monitoring of faecal coliforms and enterococci on beaches has been spatially concentrated in the Sydney and Illawarra regions, commencing on Sydney beaches in 1966 and on Illawarra beaches in 1996. Monitoring of faecal contamination on beaches and in waterways between Ballina (far north coast of NSW) and Mystery bay (far south coast of NSW) did not commence until 2002.

Associations between water quality parameters and (i) mortality events and (ii) prevalence and intensity of *Perkinsus* sp. infections in abalone were not explored further in this project. Ideally, to examine such associations, parameters of water quality would be available across the geographic range of interest and available both prior to and since the documented mortality events. Specifically, for our purposes, the available water-quality data:

(i) do not cover the spatial scale of reported mass mortalities (Port Stephens, Central Coast, Sydney, Wollongong, Kiama) and the sudden decline in abundance at Jervis Bay)

(ii) do not cover the spatial scale of *Perkinsus* sp. infections (locations: North Coast, Crowdy Head, Port Stephens, Central Coast, Sydney, Wollongong, Kiama, Jervis Bay, Merimbula)

(iii) do not cover the temporal scale required (pre and post mortalities and/or detection of *Perkinsus* sp.) for any of these locations except Sydney (where a limited range of parameters of water-quality were monitored prior to 1995).

Table 6.2. Main sources of water quality data for the NSW ocean waters since 1942, listed by category (Table 6.2.1 Location-specific monitoring pre-1990s; Table 6.2.2 Location-specific monitoring post-1990s; Table 6.2.3 Beaches and waterways – faecal contamination).

Table 6.2.1. *Location-specific monitoring, pre-1990's*

Source	Locations	Years	Parameters	Sampling frequency	Reference
CSIRO Division of Fisheries and Oceanography	Port Hacking	1942 - 1988	Temperature Salinity Dissolved Oxygen Inorganic phosphate Nitrate Nitrogen	~ weekly	MHL, 1988
Caldwell-Connell Engineers	Sydney (offshore): Turimetta Head to Marley Head	1972 - 1979	Temp Salinity Density Dissolved Oxygen Total phosphate Nitrate nitrogen TKN nitrogen Ammonia pH Water clarity	-	MHL, 1988
CSIRO Division of Fisheries and Oceanography	Stanwell Park	1983 - 1984	Temp Salinity	-	MHL, 1988
Water Board's Scientific Services	Malabar (offshore)	1987	Temp Conductivity Dissolved Oxygen pH	-	MHL, 1988

Table 6.2.2. *Location-specific monitoring, post-1990's.*

Source	Locations	Years	Parameters	Sampling frequency	Reference
Water Board's Scientific Services and State Pollution Control Commission	Sydney (inshore & offshore): Long Reef to Port Hacking	1989 - 1993	Temp Salinity Dissolved Oxygen Turbidity Suspended solids Chlorophyll-a Ammonia-nitrogen Oxidised nitrogen Inorganic phosphorus Total phosphorus Faecal Coliforms	~ every 2-3 weeks	EPA, 1995
Department of Environment and Conservation	NSW coast (Ocean STPs): North Head to Kiama	1995 - 2004	Temp Conductivity Dissolved Oxygen pH Ammonia-nitrogen Oxidised nitrogen Total phosphorus Total Kjeldahl nitrogen Total nitrogen Range of metals, pesticides & effluent toxicity	~ monthly	SWC, 2005
Department of Environment and Conservation	NSW coast (Ocean STPs) Bondi, Malabar, North Head, Cronulla, Bellambi, Port Kembla	1995 - 2004	(as above)	daily but, ~ every 6 days for Cronulla	SWC, 2005

Table 6.2.3. *Beaches and waterways, faecal contamination.*

Source	Locations	Years	Parameters	Sampling frequency	Reference
Water Board's Scientific Services and Environmental Protection Authority	Sydney (beaches): Palm Beach to Cronulla	1966 - 1993	Beach conditions Faecal coliforms Faecal streptococci Grease	~ every 6 days up to 1988; thereafter ~ weekly	MHL, 1988; & EPA, 1995
Department of Environment and Conservation	Sydney (beaches): Palm Beach to Cronulla	1994 - present	Faecal Coliforms Enterococci	~ every 6 days	SWC, 2005
Sydney Water Corporation	Illawarra (beaches): Warilla to Fisherman's Beach	1996 - present	Faecal Coliforms Enterococci	~ every 6 days	SWC, 2005
Department of Environment and Conservation - Beachwatch Partnership Pilot Program involving 15 local councils	NSW coast (beaches, waterways): Seven Mile Beach (nth of Ballina) to Maroubra; & Tilbury Cove (Shoalhaven Bight) to Mystery Bay;	2002 - 2003	Faecal Coliforms Enterococci	~ weekly	DEC, 2004

6.4. Discussion

6.4.1. *Evaluation of hypotheses regarding alternative causes of observed mass mortalities of abalone and perkinsosis*

Based on analyses presented in this and previous chapters of this report, likelihoods of alternative components of sufficient cause complexes (hypotheses presented in Section 6.1.3) for the observed mass mortalities and perkinsosis in abalone in NSW are summarised in Table 6.3. The rationale for the relative likelihoods are explained below.

Table 6.3. Hypotheses regarding component factors of sufficient cause complexes for the documented mass mortalities of abalone.

Hypothesis / Component factors in sufficient cause complex	Likelihood
Epi-Hyp-1 A pathogen (other than *Perkinsus* sp.) is necessary and sufficient	Low
Epi-Hyp-2 Water temperature (above some threshold) is necessary and sufficient	Low
Epi-Hyp-3 An environmental factor other than water temperature (above some threshold) is necessary and sufficient	Low
Epi-Hyp-4 *Perkinsus* sp. is a necessary factor	**High**
Epi-Hyp-4.1 (*Perkinsus* sp. necessary) *P. olseni* is sufficient	Low
Epi-Hyp-4.2 (*Perkinsus* sp. necessary) A pathogen (other than *P. olseni*) is a component factor	Low
Epi-Hyp-4.3 (*Perkinsus* sp. necessary) An environmental factor other than water temperature (above some threshold) is a component factor These factors are sufficient	Low
Epi-Hyp-4.4 (*Perkinsus* sp.necessary) Water temperature (above some threshold) is a component factor	**High**
Epi-Hyp-4.4.1 (*Perkinsus* sp. necessary) Water temperature (above some threshold) is a component factor These factors are sufficient	**Medium**
Epi-Hyp-4.4.2 (*Perkinsus* sp. necessary) Water temperature (above some threshold) is a component factor Other (unidentified) factor(s) involved	**Medium**

Epi-Hyp-1 and Epi-Hyp-4.2

The hypotheses that involve a pathogen other than *Perkinsus* sp. as a component factor represent unlikely explanations. Apart from *Perkinsus* sp., no other pathogens were detected by histological examination of the 209 abalone examined in this study or the 407 wild abalone from the NSDA survey in 2002-03 (Chapter 5; Callinan and Landos, 2006). Neither were any pathogens other than

Perkinsus sp. identified in abalone that were collected and examined at the time of mortality events at Terrigal in 1992, Sydney-Wollongong in 1993 and Kiama in 1996 (Section 4.1.2, Appendices 12.4.1 – 12.4.5).

Epi-Hyp-2

It is not likely that water temperature (elevated or above some threshold) is a necessary and sufficient cause. At each of the locations where mortality events were identified, there were years prior to the observed mortality events in which water temperature was equal to or greater than that during the mortality event (Section 6.3.8.3 and Fig. 6.8). If elevated water temperature, independent of *Perkinsus* sp., was the cause of mortality events then there would have been mortalities prior to and since those that were reported.

Epi-Hyp-3

It is deemed unlikely that any environmental stressor is both necessary and sufficient. During interviews, divers associated several factors with declines in abundance of abalone over time scales of the order of a year, namely severe storms (encompassing floods, large seas, sand-up events), water quality (associated with sewage treatment plants); elevated water temperature; and intensive harvesting (Section 6.3.7; see comments in Table 3.1; Section 3.4.1). Without doubt, weather-driven mortality events and other factors (e.g., poor water quality) can impact abalone stocks, causing mortalities from time to time. However, there were no environmental factors that were associated by divers with mortality events that were consistent across the mortality events identified in Section 3.3.1: the "2000 mortality event" at Port Stephens; the "1992 mortality event" on the Central Coast; the "1993 mortality event" at Sydney-Wollongong; the "1996 mortality event" at Kiama; and the 2000 – 2002 sudden decline in abundance at Jervis Bay.

Unfortunately, available data characterising water quality were of limited use for the purpose of correlating parameters of water quality with the timing of abalone mortality events and the prevalence and intensity of *Perkinsus* sp. infections in abalone. The available time-series (Section 6.3.9) do not cover the spatial and temporal scales at which abalone mortalities and detection of *Perkinsus* sp. have been documented.

Based on these arguments, it is not possible to rule out the importance of some environmental stressor as a component of a sufficient cause complex based on these arguments. However, given the evidence that the presence of *Perkinsus* sp. is a necessary factor (see below), the likelihood that an environmental stressor alone is necessary and sufficient is extremely low.

Epi-Hyp-4

The evidence that the presence of *Perkinsus* sp. is a necessary factor in a sufficient cause complex for the documented mortality events is compelling. Note that, by definition, the presence of *Perkinsus* sp. must be a necessary factor for the development of perkinsosis.

Perkinsus spp. including *Perkinsus* sp. are infectious pathogens of molluscs that have been associated with mass mortalities of shellfish worldwide (Chapter 4; Villalba *et al.*, 2004).

Perkinsus sp. infections were identified in dead and moribund abalone collected within the immediate area at the time of mass mortality events at Terrigal in 1992, Sydney-Wollongong in 1993 and Kiama in 1996 (Section 4.1.2, Appendices 12.4.1 – 12.4.5).

The geographic range of *Perkinsus* sp. infections identified in the 2005 survey was restricted to Region 1 of the abalone fishery (north of location "South Jervis Bay") with a single exception, the "outpost" of infection at a single site (Merimbula) within location "Eden-Tathra" on the far south

coast of NSW (Chapter 4). With the exception of the Merimbula site, the geographic distribution of *Perkinsus* sp. identified in 2002-03 and 2005 corresponds with the distribution of documented mass mortalities (Chapter 3). Moreover, within Region 1 between Port Stephens and Jervis Bay, there have been no reported mass mortalities of abalone at locations where *Perkinsus* sp. was not, or has not since been, identified.

Perkinsus sp. infections detected by histology in samples from both the 2002-03 and 2005 surveys were generally systemic (Chapter 5; Callinan and Landos, 2006). In abalone with high-intensity infections, there was evidence of substantial tissue and organ damage including disruption of the gut epithelium and infarction in the gills. The abalone from which these tissues were sampled were alive when collected but it is only a small extension in logic to conclude that progression of such intense infections would result in mortality (Chapter 5). Furthermore, the proliferation of *Perkinsus* spp., progression into multiple organs, functional impairment and subsequent mortality is well described in other hosts (Villalba *et al.*, 2004, Park *et al.*, 2006b).

It is concluded very likely that *Perkinsus* sp. was involved as a necessary factor in the documented mass mortalities of abalone. Given this conclusion, it is necessary to consider the alternative hypotheses that include *Perkinsus* sp. as a necessary factor.

Epi-Hyp-4.1

Whilst the presence of *Perkinsus* sp. is a necessary factor for the development of perkinsosis and it is considered "high likelihood" that it is necessary factor for the documented mortalities of abalone along the NSW coast, it is unlikely that, acting alone, it represents sufficient cause. Despite the presence, in 2005, of *Perkinsus* sp. within locations between the north coast of NSW and Jervis Bay and at Merimbula on the far south coast (Chapter 4), there have been no reported mass mortalities evident at any locations on the NSW coast since the 1996 mortality event at Kiama and the sudden decline in abundance at Jervis Bay in 2000 – 2002 (Chapter 3). Thus, whilst the presence of *Perkinsus* sp. is a necessary factor, there must be other component factors for mortalities to occur. This is not surprising, given the evidence that prevalence and intensity of *Perkinsus* spp. infections in other mollusc hosts is related to environmental variables, water temperature and salinity in particular (Sections 6.1.1 and 6.1.2).

Epi-Hyp-4.3

It is possible that some environmental variable (other than temperature), in addition to the presence of *Perkinsus* sp., was responsible for the documented mass mortalities and high-grade infections observed in abalone surveyed in 2002 and 2005. The proximity of the locations of documented mass mortalities to major coast population centres in NSW (Newcastle, Sydney, Wollongong) suggests the possibility that an environmental factor associated with the presence of such population centres (e.g., a pollutant) may be a component of a sufficient cause complex.

However, as explained for Epi-Hyp-3, there were no environmental factors that were consistently associated by interviewed divers with mortality events that were consistent across the documented mass mortality events. Secondly, available time-series of water quality data do not cover the spatial and temporal scales at which abalone mortalities and detection of *Perkinsus* sp. have been documented. Thus, identification of such an environmental factor is problematic.

However, based on the strong evidence for water temperature as a component factor in the cause of perkinsosis and host mortality, this hypothesis (Epi-Hyp-4.4) which excludes water temperature as a factor, is graded as "Low" likelihood.

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Epi-Hyp-4.4

There is strong evidence that, in addition to the presence of *Perkinsus* sp., water temperature (above some threshold) is a component factor in a sufficient cause complex. Note that the factor "water temperature (above some threshold)" is deliberately a generic statement. As well as including the concept of absolute temperature, the threshold also must include the concept of the duration of exposure to given temperatures. For example, the threshold for development of an intense *Perkinsus* sp. infection in an individual might be water temperature in excess of 20°C for 4 months. The threshold for mortality to result might be water temperature in excess of 20°C for 6 months. It is beyond the capacity of this project to assess what the actual threshold might be. Rather, we are concerned with the more general concept that some threshold associated with water temperature is a factor in disease development and mass mortalities. It is also important to note the evidence from other studies that transmission of *Perkinsus* spp. among hosts is density-dependent and development of perkinsosis within individual hosts is dose-dependent (see Section 6.1.1). Thus, the necessary factor "presence of *Perkinsus* sp." should be more specific and include the concept of some threshold dose. The clarification is important because low densities of abalone in an area accompanied by low prevalence of *Perkinsus* sp. may result in low rates of transmission and sub-threshold dosages of *Perkinsus* sp. to abalone.

Field observations and in vivo and in vitro experiments in the laboratory, both internationally and in Australia) demonstrate the temperature-dependence of mortalities due to *P. marinus* and *P. olseni / P. atlanticus* in mollusc hosts. A recurring conclusion from these investigations is that water temperatures above approximately 20°C are associated with increased or maximum rates of zoospore production, prevalence and intensity of infections, disease transmission and host mortality (see Sections 6.1.1 and 6.1.2). Results from limited laboratory experiments done in Australia concerning *P. olseni* infections in greenlip and blacklip abalone also suggest increased prevalence and intensity of infections at temperatures of approximately 20°C compared to lower temperatures (see Section 6.1.2).

At all locations on the NSW coast where mass mortalities of abalone were documented (Chapter 3), 1-year moving average SSTs were greater than 20°C (Section 6.3.8). Mean SSTs during October – June (quarters 4, 1 and 2) were greater than 21°C and mean SSTs of greater than 20°C during both January – March and April – June quarters were associated with mortality events at each location (Section 6.3.8).

Mean annual SSTs and mean quarterly SSTs declined with increasing latitude along the NSW coast (Section 6.3.8; Fig. 6.6) and no mass mortality events or sudden unexplained declines in abundance have been documented south of Jervis Bay (Chapter 3). In particular, there have been no reports of mass mortalities in the Merimbula area, nor evidence of any sudden decline in abundance in the Merimbula area (Section 6.3.6, Fig. 6.4) – Merimbula being the single site to the south of South Jervis Bay on the far south coast of NSW at which *Perkinsus* sp. has been detected. Intensity of *Perkinsus* sp. infections detected at Merimbula were all low-grade (Section 4.3.5 and Table 4.3). During the 3 years 2004 – 2006 mean annual SST for the far south coast was 18.7°, with mean SST during January – March 21.6°, April – June 18.9°, July – September 16.2° and October – December 17.8°. These temperatures were significantly lower than temperatures further north on the coast. Thus, it is plausible (but no demonstrated) that infections of *Perkinsus* sp. in abalone in colder water on the far south coast of NSW cannot develop into high-grade infections nor result in mass mortalities.

Intensity of *Perkinsus* sp. infections and tissue damage were greater in samples of abalone from the 2002 survey compared to the 2005 survey (Chapter 5). Water temperatures were greater in 2002 compared to 2005 by approximately 1°C (Section 6.3.8.4 and Fig. 6.7). These results are consistent with water temperature as a contributing factor determining disease progression.

Future repeats of the 2005 broad-scale survey of infection prevalence and intensity would provide a means of monitoring any expansion of the geographic range of *Perkinsus* sp. In particular, identification of high-intensity infections on the south coast of NSW and/or documented mortalities of abalone would provide further insight into the importance of a threshold water temperature. Replicated, controlled laboratory experiments designed to test hypotheses about the transmission, prevalence and intensity of *Perkinsus* sp. infections across a range of temperatures and (e.g., 15°C, 20°C 25°C) and exposure times (e.g., 1 week, 1 month, 3 months, 6 months) would be particularly relevant (see Chapter 8).

Epi-Hyp-4.4.1

This hypothesis (*Perkinsus* sp. and high water temperature being sufficient cause) cannot be considered as high-likelihood because of the finding that *Perkinsus* sp. infections in abalone at locations "Crowdy Head" and "North Coast" (to the north of Port Stephens) were low-intensity infections (Ray's grade ≤ 1). Under this hypothesis, the expectation is that infections would have been at least as intense as those detected between Port Stephens and Jervis Bay. Nor have there been any reports of mass mortalities of abalone from "North Coast" or "Crowdy Head" locations.

However, this hypothesis cannot be discounted and considered low likelihood because of plausible mitigating arguments. Firstly, reports of mass mortalities at these locations are less likely than at other locations to the south due to the relatively low fishing effort by abalone divers at these locations. Secondly, the finding of extremely high prevalence of low-grade infections at location "North Coast" in 2005 is also consistent with arrival of *Perkinsus* sp. during early 2005, immediately prior to the 2005 survey. In this case, introduction of *Perkinsus* sp. into a naïve population of abalone may result in high prevalence but with insufficient time for the intensity of infections to develop into high-grade infections and cause host mortality. Future sampling of the abalone population within location "North Coast" to determine the intensity of infections in abalone would provide a test of this hypothesis (see Section 7.3).

Epi-Hyp-4.4.2

Evidence for water temperature as a component of a sufficient case complex, in addition to the presence of *Perkinsus* sp., is strong (Epi-Hyp-4.4). However, given the evidence associated with Epi-Hyp-4.4.1 (above) of low intensity *Perkinsus* sp. infections in abalone at locations "North Coast" and "Crowdy Head", it is possible that other factors must also be present for development of perkinsosis into a high-grade infection with associated tissue destruction and mass mortality events.

6.4.2. *Hypotheses about the historical distribution and spread of Perkinsus* sp. *on the NSW coast*

It has been concluded (Section 6.4.1) that, with "High" likelihood, the presence of *Perkinsus* sp. was a necessary factor and water temperature (elevated or above some threshold) was a component factor involved in the documented mass mortalities of abalone and for the development of high-grade perkinsosis. It was not possible to distinguish between alternative hypotheses that excluded and included an additional unknown environmental factor (Epi-Hyp-4.4.1 and Epi-Hyp-4.4.2) in the sufficient cause complex.

Because these alternative hypotheses each involve the presence of *Perkinsus* sp. and at least one additional environmental factor as component factors within sufficient cause complexes, it is important to consider whether *Perkinsus* sp. has been endemic and widely distributed along the coast of NSW since many years prior to the epizootics. Alternatively, the documented mass mortalities of abalone and more recent detection of *Perkinsus* sp. at particular locations at

particular times may be closely associated with the arrival of *Perkinsus* sp. at these locations. Note that this would not be an issue if we had concluded that the presence of *Perkinsus* sp. alone was necessary and sufficient for the development of perkinsosis and mortality of abalone. Evidence for the 2 alternative hypotheses is considered here.

Hist-Distribn-Hyp-1:

> *Perkinsus* sp. has been endemic along the coast of NSW since many years prior to the documented mass mortalities and identification of *Perkinsus* sp. in abalone (and that spatial and temporal variations in an environmental factor determined the spatial and temporal distribution of detectable *Perkinsus* sp., pathogenicity and subsequent mortalities).

Suppose that *Perkinsus* sp. has been endemic and widely distributed along the NSW coast for many years prior to the documented mortalities and it is temporal variation in some other environmental factor that determines the temporal distribution of detectable *Perkinsus* sp., pathogenicity and subsequent mortalities. Under this hypothesis, *Perkinsus* sp. would be widely distributed along the NSW coast during both 2002 and 2005 (in addition to all other years). During each of these years, abalone populations were surveyed and tissue samples from large numbers of animals were tested for *Perkinsus* sp. using Ray's test and lesser numbers were examined histologically and tested by PCR. No *Perkinsus* sp. infections were detected in abalone from vast stretches of the NSW coastline during both surveys. For example, of 280 abalone sampled from locations Ulladulla, North Bateman's Bay and South Bateman's Bay during the 2005 survey, no *Perkinsus* sp. were identified by Ray's test on gill tissue (n = 280) or by histological examination of multiple organs (n = 15). If *Perkinsus* sp. infections in abalone existed along the entire NSW coastline during 2002 and 2005, then these infections must have existed at undetectable levels: (i) in gill tissue that was examined by Ray's test or (ii) in organs other than gill tissue at levels that were not detectable by histology. Given that a large proportion of the gill tissue was used for Ray's test (approx. 50%) and single *Perkinsus* sp. cells are easily detected, it is unlikely that *Perkinsus* sp. was present at undetectable levels in the gill tissue. In contrast, low-level infections (Ray's grade $\leq$ 1) were rarely detected by histology (Section 4.3.1 and Fig. 4.3). Thus, if low-level infections of *Perkinsus* sp. exist in organs other than gills, it is likely they will not be detected by the methodology used in this project. When, however, *Perkinsus* sp. infections were detected by histology in this project, the gills were one of several organs within which *Perkinsus* sp. was most frequently found (Section 5.3.1 and Fig. 5.1). While possible, it seems unlikely that *Perkinsus* sp. infections existed in abalone sampled from the stretches of coastline in 2002 and 2005 for which no infections were detected. This suggests that it is unlikely that *Perkinsus* sp. has been endemic and widely distributed along the coast of NSW since many years prior to the epizootics.

The detection of *Perkinsus* sp. in abalone at the Merimbula site during the survey in 2005 is also inconsistent with this hypothesis. Under this hypothesis, it is spatial and temporal variation in an environmental factor that determines the spatial and temporal distribution of detectable *Perkinsus* sp., pathogenicity and subsequent mortalities. That *Perkinsus* sp. was detected in 4 of the 20 abalone sampled from the Merimbula site in 2005, but not detected in any of the 321 abalone sampled from sites between South Jervis Bay and Merimbula, or in any of the 61 abalone sampled from sites to the south of Merimbula is inconsistent with this hypothesis. Detection of *Perkinsus* sp. in abalone at Merimbula would imply the presence of an environmental stressor at this site that is simultaneously absent from the other 19 sites to the south of South Jervis Bay.

It is therefore concluded, from available evidence, as unlikely that *Perkinsus* sp. has been endemic along the coast of NSW since many years prior to the documented mass mortalities and identification of *Perkinsus* sp.

As a component of future surveys, application of a suitably sensitive testing methodology for the identification of *Perkinsus* sp., across multiple organs would provide further evidence for the presence/absence of *Perkinsus* sp. infections in abalone sampled from sections of the NSW coast for which infections were not detected during the 2005 survey. As discussed in Section 4.4.1 and Section 7.3, further development of real-time PCR assays accompanied by a formal study of the comparative sensitivity of real-time PCR, histology and Ray's test would be valuable. An established real-time PCR with sensitivity equivalent to Ray's test, but with application to tissue samples from multiple organs, would facilitate fast and efficient testing for the presence of *Perkinsus* sp. in multiple tissue samples from multiple organs from multiple abalone sampled from survey sites.

Hist-Distribn-Hyp-2:

> *Perkinsus* sp. has not been endemic to the entire coast of NSW for many years prior to the documented mass mortalities and identification of *Perkinsus* sp. in abalone (and that detection of *Perkinsus* sp. in abalone at a location at a point in time is closely associated with the arrival of *Perkinsus* sp.*)*.

The alternative hypothesis to *Epi-HypHistorical-Distribution-A* is that documented mass mortalities of abalone and more recent detection of *Perkinsus* sp. at particular locations at particular times occurs soon after the arrival of *Perkinsus* sp. at these locations (*Epi-Hyp-Historical-Distribution-B)*. Note that the development of high-grade perkinsosis and subsequent mortality would only occur if the auxiliary environmental variable(s) exceeds the necessary threshold(s). The spatial and temporal pattern of documented mass mortalities (Chapter 3) is consistent with this hypothesis.

Consider a scenario in which *Perkinsus* sp. was introduced into the vicinity of the Central Coast/Sydney-Wollongong in the early 1990s, by an unknown process from an unknown source. This pattern of documented mortality events is then consistent with the spreading of *Perkinsus* sp. through naïve populations of abalone to the north and south of the Central Coast/Sydney-Wollongong since the early 1990s. Mortality events occurred on the Central Coast in 1992 and at Sydney-Wollongong in 1993. This was followed, further to the south, by the "1996 mortality event" at Kiama and subsequently, further to the south, the "2000 – 2002 sudden decline in abundance" at Jervis Bay. The "1992 mortality event" on the Central Coast was followed by the "2000 mortality event" at Port Stephens to the north.

Extension of the documented range of *Perkinsus* sp. infections occurred between 2002 and 2005, to the north as far as Point Perpendicular (within location "North Coast") and to the south at the single "outpost" of infection at Merimbula (location Tathra-Eden). These extensions of documented range are consistent with, but do provide substantial evidence for this hypothesis because the 2002 survey didn't extend to the north of Crowdy Head, nor to the south of location "South Bateman's Bay" (i.e., not as far south as the Merimbula site at which *Perkinsus* sp. was detected in 2005). It cannot be effectively demonstrated that *Perkinsus* sp. infections in abalone did not exist at these locations in 2002.

Whether transmission of *Perkinsus* sp. among locations occurred by passive or active mechanisms (see discussion in Section 6.4.3), *Perkinsus* sp. must, under this hypothesis, have arrived at a location sometime prior to the documented mortality event. Under *Epi-Hyp-4.4.1* (the combination of *Perkinsus* sp. and water temperature above some threshold being necessary and sufficient), development of high-grade perkinsosis and mortality would occur as soon after the arrival of *Perkinsus* sp. as water temperature exceeded its threshold for a sufficient amount of time. Under *Epi-Hyp-4.4.2* (*Perkinsus* sp., temperature stress above threshold and some other environmental variable above threshold being necessary and sufficient) development of high-grade perkinsosis

and mortality would occur as soon after the arrival of *Perkinsus* sp. as both water temperature and the other environmental variable exceeded threshold values. If development of high-grade perkinsosis requires water temperature to be above a threshold of approximately 20°C (Section 6.3.8), for a period of, say 6 months, then this threshold was exceeded for each of the documented mortality events. This threshold was not exceeded at Merimbula in 2005, consistent with only low-grade *Perkinsus* sp. infections being detected here at this time. This threshold was exceeded, however, for locations "North Coast" and "Crowdy Head" in 2005 where *Perkinsus* sp. infections were only of low-grade intensity (see discussion re *Epi-Hyp-4.2.1* and *Epi-Hyp-4.2.2* in Section 6.4.1).

Another observation that is consistent with the spreading of *Perkinsus* sp. through naïve populations of abalone are the observations of maximum prevalence of *Perkinsus* sp. infections at the northern and southern boundaries of the documented geographic range of *Perkinsus* sp. This observation was originally based on the 2002 survey data (Callinan and Landos, 2006; and see Appendix 6 in Section 12.6). In 2005, this pattern was repeated on the north coast of NSW with infections most prevalent at 3 sites within location "North Coast". On the south coast, excluding the "outpost" of *Perkinsus* sp. infection at Merimbula, St. Georges Head (South Jervis Bay) was the most southern site at which *Perkinsus* sp. infections were identified. Prevalence of infections at this site was greater than at the sites to the north of St. Georges Head within South Jervis Bay, at which infections has been most prevalent in 2002 (Section 4.4.3, Table 4.3, Fig. 4.5).

Considering the consistency of available evidence with the 2 hypotheses about the historical distribution and spread of *Perkinsus* sp. on the NSW coast it is concluded that the most probable explanation is that the observed mortalities and/or detection of *Perkinsus* sp. in abalone at a location at a point in time is closely associated with the arrival of *Perkinsus* sp. That is, *Perkinsus* sp. was not endemic to the entire coast of NSW for many years prior to the documented mortality events and subsequent detection of *Perkinsus* sp.

6.4.3. Mechanisms of transmission

Transmission of *Perkinsus* spp. cells (trophozoites and/or hypnospores and/or zoospores) via ocean currents and the potential for active transmission by vectors including other marine species and human beings through their activities has been demonstrated (see Section 6.1.1). The scope of this project did not include experiments or extensive surveys concerning mechanisms of transmission. This project did, however, include a small-scale survey of the presence of *Perkinsus* sp. infections in alternative mollusc hosts at 3 sites in the vicinity of Jervis Bay (Sections 6.2.4 and 6.3.4) and evaluation of the potential for transmission of *Perkinsus* sp. through translocation of abalone during fishing operations (Section 6.3.5) and through translocation/reseeding experiments (Section 6.3.6).

No *Perkinsus* sp. infections were detected in any of the 4 mollusc species (total $n = 176$) from the small-scale survey to identify alternative mollusc hosts for *Perkinsus* sp. at Jervis Bay. Neither did Gill (2003) detect *Perkinsus* sp. infections among the 7 specimens sampled from "Drum and Drumsticks" at North Jervis Bay or the 190 specimens sampled at Wanda Wanda Head near Port Stephens in 2003. *Perkinsus* infections have been found in many species in other locations around Australia and, in particular, 9 species including blacklip and greenlip abalone around the Yorke Peninsula in South Australia (Goggin and Lester, 1995). Moreover, *Perkinsus* isolated from 8 mollusc species were shown to have low host specificity in the laboratory (Goggin *et al.*, 1989). In other studies, transmission of *Perkinsus* spp. has been demonstrated both with and without alternative / intermediate hosts (Section 6.1.1). The limited number of alternative mollusc hosts and sites and locations surveyed in NSW therefore prevents the conclusion that *Perkinsus* sp. does not infect species other than blacklip abalone in NSW. The only statement that can be made is that no alternative host species for *Perkinsus* sp. have yet been identified in waters along the NSW coast. Thus, the involvement of intermediate hosts in the transmission of perkinsosis along the

NSW coast cannot be discounted. Sampling of alternative mollusc species as a component of future broad-scale surveys of *Perkinsus* sp. infections in abalone would provide more conclusive evidence regarding alternative hosts (see Section 7.3).

Multiple activities were identified that involved translocation of abalone during fishing operations that could have in the past and in the future result in transmission of *Perkinsus* sp. Activities identified that involved translocation of abalone at spatial scales up to 10's of km included:

- harvesting of abalone by divers at individual drops (10's – 100's metres)
- hanging of abalone over the side of the boat or pumping water over abalone in the boat during a day's fishing (10's km)
- hanging of catch during multi-day trips (10's km – approx. 100 km)
- movement of sub-legal abalone among reefs (10's of km)
- shucking of abalone and discard of viscera (10's km)

Activities associated with fishing that involved translocation of abalone at greater spatial scales included:

- movement of divers, boats and equipment between locations (100's km)
- movement of catches to processors and distributors (100's km)
- distribution of abalone viscera as bait (100's – 1000's km)

It is also possible that experiments involving the translocation and reseeding of abalone between 1999 and 2001 could have inadvertently translocated abalone carrying *Perkinsus* sp. infections (Sections 6.2.6 and 6.3.6). Brood-stock for the abalone hatchery involved in these experiments was obtained from wild populations of abalone at locations at which *Perkinsus* sp. infections were known to occur. As there was no testing of samples of brood-stock or hatchery-reared juveniles prior to 2002, the possibility that hatchery-reared juveniles and larvae released at sites along the NSW coast were infected with *Perkinsus* sp. could not be excluded. Some of these releases were into locations within which *Perkinsus* sp. was known to already exist (Sydney and Kiama). Releases in the vicinity of Port Stephens between 1998 and 2001 were prior to the first identification of *Perkinsus* sp. at Port Stephens in 2002 but coincident with the "2000 mortality event" at Port Stephens. Releases of hatchery-reared juveniles at Tura Pt, Merimbula in 2001 and at sites near Eden between 1999 and 2001 were at locations at which *Perkinsus* sp. was not known to occur.

It has been discussed that the pattern of documented mortality events is consistent with the spreading of *Perkinsus* sp. through naïve populations of abalone to the north and south of the Central Coast/Sydney-Wollongong since the early 1990s (Section 6.4.2). The spatial and temporal pattern of documented mortality events (at Central Coast in 1992, Sydney-Wollongong in 1993, Kiama in 1996 and the sudden decline in abundance at Jervis Bay during 2000 – 2002) is consistent with mechanisms of passive transmission, especially given the prevailing southerly currents associated with the East Australia Current (EAC). Similarly, the "2000 mortality event" at Port Stephens, to the north of Central Coast and Sydney-Wollongong is not inconsistent with passive transmission given the variability of eddies (both anti-clockwise warm-water and clockwise cold-water eddies) associated with the EAC, northerly inshore currents and longshore transport on this section of the NSW coast (e.g., Cresswell *et al.*, 1983; Nilsson and Cresswell, 1981; Marchesiello and Middleton, 2000). Passive transmission *Perkinsus* sp. cells (trophozoites and/or hypnospores and/or zoospores) could also have been supplemented by any of the active mechanisms of transmission that have been identified.

If the detection of *Perkinsus* sp. at locations to the north of Port Stephens ("Crowdy Head" and "North Coast") in 2005 represents an expansion of the range of *Perkinsus* sp. since 2002, then transmission may have resulted from passive or active mechanisms. Note, however, that there was

no identification of *Perkinsus* sp. at sites within location Forster – Seal Rocks in 2002 or 2005 and this location is between Port Stephens – Broughton Island and the Crowdy Head. This suggests an increased likelihood of active mechanisms of transmission.

Whether or not the main mechanism of transmission of *Perkinsus* sp. among locations between "North Coast" and "South Jervis Bay" was passive rather than active, passive transmission cannot readily explain the presence of *Perkinsus* sp. at one isolated site on the far south coast of NSW (Merimbula) by 2005. This is of course assuming that *Perkinsus* sp. was not present in abalone at undetectable levels at the many sites between South Jervis Bay and Merimbula that were sampled in 2005. Any of the active mechanisms of transmission identified previously may have been involved (other marine species, birds, human activities). However, those that appear most likely are those fishing-related activities that operate at the scale of 100's of km (movement of divers, boats and equipment between locations; distribution of abalone viscera as bait) and the translocation of potentially-infected abalone to Tura Pt (5 km to the north of the Merimbula site) that occurred in 2001 during translocation/reseeding experiments.

As an adjunct to any future survey of the prevalence of *Perkinsus* sp. infections in abalone along the NSW coast, it would be useful to include additional survey sites at and in the vicinity of sites at which juvenile abalone were released during the reseeding experiments during 1999 – 2001 (Tura Pt near Merimbula and several sites near Eden). This would facilitate testing the hypothesis that *Perkinsus* sp. infections exist in abalone at sites in close proximity to reseeding sites but do not exist at sites away from reseeding sites (see Section 7.3).

Other important aspects of the epizootiology of perkinsosis have also been addressed in this study. Conclusive evidence for the primary portals of entry for *Perkinsus* spp. into hosts is lacking but several studies have suggested the importance of the gill and labial palps (see Section 5.1). This project did not involve specific experiments to address this issue but findings from the histology work are consistent with the likely importance of the gill as a primary portal for entry of *Perkinsus* sp. into abalone. Firstly, of the various organs within which *Perkinsus* sp. trophozoites and tomonts were found, gill tissue was one of the most frequently infected. Secondly, in 4 of the 6 instances that *Perkinsus* sp. was detected in a single organ, the organ affected was the gill (Section 5.3.1). However, other scenarios including ingestion could not be ruled out.

Perkinsus sp. infections were more frequent in female (10.9%) than in male (5.0%) abalone but no significant difference in the intensity of infections between males and females sampled during the 2005 survey (Section 6.3.1). Equivalent analysis of data from the 2002 survey showed no significant difference in the prevalence of infections in males (8.3%) and females (10.0%). The significance of the greater prevalence of *Perkinsus* sp. infections in females than males during 2005 is not known and we could find no precedence in the literature for gender-specific prevalence.

Prevalence and intensity of *Perkinsus* sp. infections did not differ significantly among the 4 size-classes (length-classes) of abalone examined (Section 6.3.2). This contrasts with several studies that found greater prevalence and/or intensity of *Perkinsus* spp. infections in adult compared to juvenile hosts. It has been speculated that this may be due to greater filtration rates and longer exposure to infective cells (Sections 5.3.1 and 5.4).

6.5. Conclusions

- Prevalence of *Perkinsus* sp. infections was gender-specific with infections more frequent in female (10.9%) than in male (5.0%) abalone in 2005. There was no gender-specific difference in infection intensity.

- Prevalence and intensity of *Perkinsus* sp. infections in abalone during 2005 was not dependent on the size (length) of abalone.

- There has been no decline in abundance of abalone at fishery-independent survey sites (monitored since 2005) adjacent to the southern "front" of infection (located at St George's Head within location South Jervis Bay in 2005).

- No alternative host species for *Perkinsus* sp. in NSW have yet been identified. No *Perkinsus* sp. infections were identified in 4 mollusc species sampled near Jervis Bay in 2005. Neither were any *Perkinsus* sp. infections identified by Gill (2003) at sites near Jervis Bay and Port Stephens.

- There were no environmental factors that were consistently associated by interviewed divers with mortality events that were consistent across the documented mass mortality events (identified in Chapter 2).

- The potential to examine associations between water quality and observed mortality events was limited. Available time-series of water quality data do not cover the spatial and temporal scales at which abalone mortalities and detection of *Perkinsus* sp. have been documented.

- The documented mortality events at specific locations in specific years were not consistently associated with increases, decreases, maximum or minimum values in any of the indices of water temperature examined. Mortality events were, however, associated with indices of water temperature in excess of 20°C (20°C, 21°C or 22°C for the 3 indices considered).

- The greater intensity of *Perkinsus* sp. infections identified in 2002 compared to 2005 corresponds with indices of water temperature that were also greater in 2002 than in 2005, consistent with the hypothesis that progression of infection intensity is related to water temperature.

- Among various hypotheses concerning factors involved in the cause of documented mass mortalities:

    - the likelihood that *Perkinsus* sp. was a necessary factor was considered "High";
    - the likelihood that water temperature (above some threshold) was an additional component of cause was considered "High"
    - the likelihood that these 2 factors were sufficient was considered "Medium"
    - the likelihood that an additional environmental factor was also involved was considered "Medium"

- Alternative hypotheses concerning the historical distribution of *Perkinsus* sp. on the NSW coast were considered and, based on consistency of hypotheses with available information, the hypothesis that:

    > "*Perkinsus* sp. has been endemic along the coast of NSW since many years prior to the documented mass mortalities and identification of *Perkinsus* sp. in abalone (and that spatial and temporal variations in an environmental factor determined the spatial

and temporal distribution of detectable *Perkinsus* sp., pathogenicity and subsequent mortalities)"

was considered less likely to be correct than the hypothesis that:

"Perkinsus sp. has not been endemic to the entire coast of NSW for many years prior to the documented mass mortalities and identification of *Perkinsus* sp. in abalone (and that detection of *Perkinsus* sp. in abalone at a location at a point in time is closely associated with the arrival of *Perkinsus* sp.*)."*

- The spatial and temporal pattern of documented mortalities (Chapter 3) and finding that prevalence of *Perkinsus* sp. infections was greatest at the boundaries of the infected zone (excluding the "outpost" of infection at Merimbula; Chapter 4) are consistent with an epizootic propagating through naïve populations of abalone.

- Multiple fishing-related activities were identified that could have translocated abalone infected with *Perkinsus* sp. and facilitated transmission of *Perkinsus* sp. into populations of abalone that were previously disease-free. Activities that involved translocations at spatial scales of less than 100's of km included: harvesting of abalone by divers at individual drops; hanging of abalone over the side of the boat or pumping water over abalone in the boat during a day's fishing; hanging of catch during multi-day trips; and shucking of abalone and discard of viscera. Activities that involved translocation of abalone at greater spatial scales included: movement of divers, boats and equipment between locations; movement of catches to processors and distributors; and the distribution of abalone viscera as bait.

- Experiments involving the translocation of abalone from the wild to a breeding facility and the subsequent translocation of hatchery-reared juveniles and larvae from the facility to the wild between 1999 and 2001 could have inadvertently translocated abalone carrying *Perkinsus* sp. infections.

- The spatial and temporal pattern of documented mass mortality events and of *Perkinsus* sp. infections between "North Coast" and "South Jervis Bay" is consistent with the passive transmission of *Perkinsus* sp. among adjacent populations of abalone but possibly supplemented by active mechanisms of transmission.

- The isolated "outpost" of *Perkinsus* sp. infection in abalone at Merimbula is likely to have resulted from an active mechanism of transmission. Such an active mechanism could have involved a mobile marine species or bird acting as a vector, the fishing-related activities that involved translocation of abalone or abalone viscera over 100's of km, or the translocation/reseeding experiments that included the release, in 2001, of hatchery-reared juvenile abalone at Tura Pt (5 km to the north of the Merimbula site at which *Perkinsus* sp. was identified in 2005).

B2

7. FURTHER DEVELOPMENT – MANAGEMENT AND RESEARCH OPTIONS

7.1. Introduction

Internationally, *P. olseni* is listed by the OIE as a notifiable pathogen and consequently, some countries may require imports of shellfish to be certified free from *P. olseni* infection (OIE, 2009). Prevention of introductions is of great importance, because outbreaks of diseases in the wild are extremely difficult to eradicate (DAAF, 2004; Villalba *et al.*, 2004). Control and prevention measures for *P. olseni*, listed by the OIE, are limited to the long-term options of chemotherapy and resistance breeding (OIE, 2009). Recent research concerning these options for *Perkinsus* spp. infections in mollusc hosts has focused on these two areas and includes selective breeding to obtain disease-resistant hosts (e.g., Ragone Calvo *et al.* 2003a; Villalba *et al.*, 2004) and chemotherapeutants for use in closed systems (e.g., Faisal *et al.*, 1999; Villalba *et al.*, 2004; Elandalloussi *et al.*, 2005; Chu *et al.*, 2006).

Within Australia, Commonwealth Aquavet plans outline the strategy for control and eradication of particular diseases in shellfish (e.g., DAFF, 2004; Handlinger, 2007). Disease response plans also exist for various diseases within each state but not specifically for *Perkinsus* sp. East (2003) highlights that there is no zoning program within Australia to keep unaffected areas free from *Perkinsus* infections. For diseases that have become widespread over time and eradication is no longer an option, then containment, control and zoning are options (e.g., DAFF, 2004). A range of control and prevention measures have been implemented by other Australian states in order to reduce the risk of spread of *Perkinsus* within abalone populations. In response to increased prevalence of *P. olseni*, a ban on shucking of abalone at sea, closures of areas affected by *P. olseni* and the removal of abalone showing clinical signs of disease from reefs have been implemented in South Australia (e.g., Casement, 2003).

A precautionary approach to the management of populations of abalone affected by *Perkinsus* sp. in NSW is warranted. Current understanding of the epizootiolgy of perkinsosis is NSW and in other Australian jurisdictions is incomplete and there is potential for further impact of the disease on the commercial and recreational fishery concentrated on the south coast of NSW. In this chapter, we consider immediate management risks and options and research and monitoring options that will inform management in the longer term.

The principal objectives of this component of the project, presented in this chapter, were to:

(i) Identify immediate management risks and options;

(ii) Identify high priority research and monitoring options that will inform management in the longer term.

7.2. Immediate management – risks and options

7.2.1. Limiting the risk of transmitting Perkinsus sp. to unaffected populations of abalone

A formal risk analysis can include the calculation of probabilities or, if information is scarce, an estimation of the likelihood for risk of disease introduction, spread and establishment, as well as the magnitude of consequences (Jones and Stephens 2006). The mechanisms of transmission of

Perkinsus sp. among hosts in NSW are poorly understood. However, a range of activities that may have been associated with the translocation and transmission of *Perkinsus* sp. among abalone populations and the spatial scale at which these activities operate have been identified (Section 6.3.5, 6.3.6 and 6.4.3). These activities also have the potential, in the future, to translocate *Perkinsus* sp. and further extend the geographic range of the *Perkinsus* sp. infections in abalone.

Whilst the geographic distribution of *Perkinsus* sp. is broad-ranging to the north of St. Georges Head (near Jervis Bay), the parasite has been identified at a single site (Merimbula) on the far south coast of NSW (Section 4.3.2). No mass mortality events associated with *Perkinsus* sp. have been identified in the vicinity of Merimbula nor is there evidence of decline in abundance of abalone at Merimbula (Fig. 6.4 in Section 6.3.6). It is also possible that transmission, progression in intensity and possibility of mortality resulting from *Perkinsus* sp. infections in abalone on the far south coast is limited by the lower ambient water temperature compared to more northern locations (Chapter 6). However, given the uncertainties about the epizootiology of perkinsosis in NSW, management options to limit the risk of transmission of *Perkinsus* sp. to unaffected populations of abalone on the far south coast of NSW are a priority.

Closures to fishing

Containment of disease outbreaks and the spread of pathogens in the environment may be achieved by closures (e.g., DAAF 2004). This approach is problematic in open marine systems where the pathogen may be widespread but may still be appropriate in cases where host species are not very mobile, such as abalones. Zoning of areas and restricting the movement of abalones between zones is consistent with the National Strategy adopted for disease outbreak situations or for areas where pathogens have become endemic but the aim is to reduce their spread to other areas (DAAF 2004).

If closures are implemented to contain parasite spread, then establishment of a buffer zone between the infected areas and disease-free areas, and active surveillance of the parasite are required (see zoning policy guidelines in: Anon., 2000; DAAF, 2004; OIE, 2009).

At present, the component of Region 1 of the abalone fishery in NSW, between Port Stephens and Jervis Bay, is closed to commercial and recreational fishing for abalone. Commercial and recreational harvesting of abalone occurs to the north of Port Stephens and in the vicinity of Merimbula, areas where *Perkinsus* sp. infections in abalone have been identified. It is important to note that the closures introduced between Port Stephens and Jervis Bay (see Section 3.1) were implemented with the primary objective of removing fishing mortality from populations of abalone that were heavily depleted. They were not specifically implemented to reduce the possibility of translocating abalone infected with *Perkinsus* sp. Furthermore, a structured fishing survey during 2007-08, to collect data about abundance of abalone within the closure involved limited harvesting of abalone by fishers. Similar surveys and/or fishery-independent surveys of abundance are planned for future years and there is the prospect of opening this component of the coast to limited commercial and recreational harvesting if and when recovery of stocks is demonstrated.

If closures were implemented with the objective of containing *Perkinsus* sp. infections, then there are several consequences. It is likely that infections of *Perkinsus* sp. in abalone within the existing closure will persist into the future, so there is minimal chance that this area would be opened to fishing in the future. Moreover, the existing closure would need to be extended to the north coast of NSW, a buffer zone implemented south of St. Georges Head (South Jervis Bay) and another closure, including buffer zones, implemented in the vicinity of Merimbula.

Fishing practices and risk of transmission

Another approach to minimising the risk of *Perkinsus* sp. transmission concerns modification of existing fishing practices that involve a risk of transmission. The range of activities, identified in

Chapter 6, that present a risk of translocating and transmitting *Perkinsus* sp. into disease-free areas, especially on the south coast of NSW, are considered here.

There were multiple activities and practices associated with fishing that involved the translocation of abalone (potentially infected with *Perkinsus* sp.) that occurred within spatial scales up to 10's of km: harvesting of abalone by divers at individual drops; hanging of abalone over the side of the boat or pumping water over abalone in the boat during a day's fishing; movement of sub-legal abalone among reefs; and the illegal practice of shucking of abalone and discard of viscera (see Section 6.3.5). Given the spatial scale of up to 10's of km, such practices will not extend the geographic distribution of *Perkinsus* sp. when diving occurs to the north of Jervis Bay. There is, however, the possibility that such practices could extend the distribution of *Perkinsus* sp. around the Merimbula area.

Hanging of catch also occurs on multi-day trips on the north coast and could transmit *Perkinsus* sp. over distances up to approximately 100 km (Section 6.3.5). Given the locations at which this practice occurs (100s of km to the north of Jervis Bay), this practice is will not result in transmission of *Perkinsus* sp. to the south coast of NSW. If, for whatever reason, hanging of abalone was to occur in the future on the south coast of NSW, there would be a risk of disease transmission between areas and consequently, this practice is undesirable.

Activities identified that involved translocation of abalone at spatial scales of 100's or 1000's of km included: the movement of divers, boats and equipment between locations; movement of catches to processors and distributors; the distribution and use of abalone viscera as bait (Section 6.3.5). The spatial scale at which these activities occur presents the risk of introducing *Perkinsus* sp. into disease-free areas.

It is possible to minimise the risk of translocating viable cells of *Perkinsus* sp. resulting from the movement of divers, boats and equipment between locations. The development of "Standard Operating Procedures" for the decontamination of equipment (tools, boats, people and clothing, transport containers) provides a means of minimising this risk (see Appendix 7, Section 12.7). These operating procedures are, however, voluntary and the current level of compliance with these procedures is not known. If, in the absence of closures, such procedures are to be the main defence against transmission of *Perkinsus* sp. into disease-free areas of the coast, then consideration should be given to making these procedures mandatory. An important issue associated with such a decision concerns the difficulties of monitoring and enforcing compliance with these procedures.

In the absence of closures preventing the harvesting of abalone potentially infected with *Perkinsus* sp., it is inevitable that abalone infected with *Perkinsus* sp. will be received by abalone processors. A review of current procedures used by processors and audit against best practice procedures were beyond the scope of this project. It is recommended as a high priority, however, that such a review is undertaken.

The distribution and use of abalone viscera as bait may result in the transmission of *Perkinsus* sp. over 100's or 1000's (inter-state) of km (Section 6.3.5). Cells of *Perkinsus* sp. can survive freezing (Goggin *et al.*, 1990), so containers of frozen "Ab. gut" sold by bait and tackle shops along the NSW coast and containing abalone potentially infected with *Perkinsus* sp. presents a risk. To counter this risk and the risk of transmitting abalone viral ganglioneuritis (AVG), the use of abalone viscera as bait or burley was made illegal in NSW in December 2007.

7.2.2. Harvesting populations of abalone depleted by Perkinsus sp.

Abalone populations between Port Stephens and Jervis Bay have been severely depleted, due to a combination of fishing and mortalities resulting from the pathogen *Perkinsus* sp. Given the absence of fishery-dependent data since the closures to commercial fishing and limited fishery-

independent survey sites in this region (Chapter 3), assessment of the status of abalone stocks is problematic.

A structured fishing survey, done in 2007, involved a limited number of commercial abalone divers fishing at specified drops (specific locations) between Port Stephens and Jervis Bay. The specified drops at which fishing occurred were selected from "historically productive drops" identified by the commercial abalone divers interviewed during this project (Section 3.2.1). Analysis of data from this survey is incomplete but preliminary analyses suggest significant variation in rates of catch among drops within zones and among zones. Mean catch rates from the Kiama area were greater than those achieved between Port Stephens and Wollongong (D. Ferrell, DII NSW, pers. comm.). Further development of this "structured fishing" survey design is currently underway.

Continued monitoring of abundances of abalone at fishery-independent survey sites also provided a means of monitoring abundance and recovery of stocks in this region up until the last year of this survey in 2007. Future repeats of this survey would provide more recent data to inform management decisions concerning access of fishers to the resource. This survey covers a limited number of locations but is a non-destructive sampling technique that does not involve the removal of abalone.

Whether abalone stocks in *Perkinsus* sp.-affected areas of Region 1 can sustain commercial harvesting has not yet been determined. In addition to controlling risks of transmission of *Perkinsus* sp., a future harvest strategy for this region will need to recognise the possibility that there may be future mortality events due to *Perkinsus* sp. in addition to mortality from future fishing. A limit reference point in terms of depletion relative to pre-exploitation biomass or biomass in a reference year would need to be more conservative given the unpredictability of future mortalities due to *Perkinsus* sp.

7.3. Research and monitoring – high priorities

7.3.1. Monitoring the geographic distribution of Perkinsus sp.

Future repeats of the broad-scale survey completed in 2005 would reveal any future expansion in the geographic range of *Perkinsus* sp. infections in abalone. Expansion of the range of *Perkinsus* sp. infections to the south of South Jervis Bay are a particular concern, given that this section of the NSW coast sustains the commercial fishery. A repeat survey (e.g., every 5 years) would facilitate ongoing monitoring of geographic range and further inform understanding of the spread of *Perkinsus* sp. infections. The geographic range of the survey should also be extended to the far north coast of NSW, given that the northern extent of the survey in 2005 was site "Bonnie Hills" (between Laurieton and Port Macquarie) within location "North Coast".

7.3.2. Monitoring of mortality events

If, in the future, any mortality events are reported by divers (commercial abalone fishers or recreational divers), identification of ambient environmental conditions (water temperature and any unusual ambient conditions) and samples of moribund abalone would be of great value. If examination of moribund abalone reveals infection with *Perkinsus* sp., then the intensity of infection and extent of tissue destruction could be associated with the condition of the abalone and associated mortality event. This was not achieved during this project because all findings about the intensity of infections and consequent tissue destruction came from abalone that were collected live, showed no clinical signs of disease prior to testing and histological examination and were not associated with any mortality event. Note that rapid autolysis generally renders dead abalone

useless for examination by histopathology, so the collection of moribund abalone from future mortality events is of fundamental importance.

During this project, the abundance of abalone at 8 sites adjacent to the southern front of *Perkinsus* sp. infection at South Jervis Bay was monitored (Section 6.2.3). Between April 2005 and October 2006, there was no significant decrease in the abundance of abalone at these sites and no observations of morbidity or mortality. Based on the proximity of these sites to the St. Georges Head site at which *Perkinsus* sp. infections were prevalent, continued monitoring of abundance of abalone at these 8 fixed sites (or in conjunctions with future repeats of the fishery-independent survey of abundance of abalone) would be valuable. Ideally, these sites would be surveyed during the period January – June when it is expected that infections of *Perkinsus* sp., if present, would be developing.

7.3.3. Development, in Australia, of real-time PCRs for the identification of Perkinsus sp.

Given the potential advantages of the speed, efficiency and specificity of PCR over Ray's test and histology for detecting *Perkinsus* infections, further development of PCR assays for diagnosis of *P. olseni* and *Perkinsus* sp.-variant ITS infections in abalone in NSW and Australia is indicated. In particular, real-time PCR can be used to more accurately quantify initial parasite levels in tissue than the standard PCR, which relies on an end-point reaction. Some pilot development work of a Taqman real-time PCR for detecting *P. olseni* in molluscs is currently being done at the CSIRO Aquatic Animal Health Laboratories in Geelong and initial results are promising (S. Corbeil, pers. comm.). The need for further development and a formal study of the comparative sensitivity of real-time PCR, histology and Ray's test is a high priority. Establishment of such a technique within Australia would provide the fast and efficient diagnosis of infections of *P. olseni*, variants of *P. olseni* and other species of *Perkinsus* in tissue samples from a range of mollusc organs. This capacity is seen as fundamental to the efficient operation of other research projects considered here.

7.3.4. The importance of water temperature as a factor in the development of perkinsosis

Uncertainty about sufficiency of the presence of *Perkinsus* sp. and water temperature elevated above some threshold (e.g., 20°C) as the cause of the development of perkinsosis and eventual mortality of abalone can be addressed in several ways.

The finding that infections of *Perkinsus* sp. in abalone sampled from "Crowdy Head" and "North Coast" in 2005 were low intensity (Ray's grade ≤ 1) infections is inconsistent with the hypothesis that the presence of *Perkinsus* sp. and water temperature above some threshold were sufficient cause for the development of high-intensity infections and subsequent mortality (Epi-Hyp-4.4.1 in Section 6.4.1). One possible explanation for this finding was that the prevalent low-intensity infections detected at "North Coast" sites resulted from the transmission of *Perkinsus* sp. to abalone at this location immediately prior to the detection of infections in 2005. Further sampling of abalone from these locations to estimate prevalence and intensity of infections would provide a test of this possibility. Given the relatively high water temperature on the north coats of NSW, if high-grade infections of *Perkinsus* sp. are not identified in abalone at this location (several years after 2005), then this would indicate that the presence of *Perkinsus* sp. and elevated water temperature are not sufficient factors for development of high-grade perkinsosis.

There is also an important role for manipulative laboratory-based experiments concerning the role of water temperature in disease progression. Experimental treatments in which abalone were exposed to *Perkinsus* sp. at temperatures between 15° and 25°C (e.g., 15°, 20°, 25°C) for various durations (e.g., 1 month, 3 months, 6 months) and subsequently tested for prevalence and intensity of infection would provide a test of the hypothesis that infection intensity, tissue destruction and mortality are positively associated with water temperature and duration of elevated water

temperature. Such an experiment would obviously include control tanks within which abalone experienced equivalent water temperatures and duration but without being exposed to *Perkinsus* sp.

Increased understanding of the importance of water temperature is fundamental to future management of the fishery given that predictions from climate change models suggest increasing water temperature on the south coast of NSW.

7.3.5. Intra-annual and inter-annual variations in prevalence and intensity of Perkinsus sp. infections

Studies of *Perkinsus* spp. in other hosts and the finding from this project that intensity of *Perkinsus* sp. infections in abalone was significantly greater in 2002 compared to 2005 (water temperature being greatest in 2002) suggest the need for further understanding of inter-annual variability. Some understanding of inter-annual variability would accrue from repeats of the 2002 survey every 5 years but an alternative approach could involve the selection of a sub-set of sites from the 2005 survey and monitoring infection prevalence and intensity in abalone at these sites more frequently (annually or every 2 years). If , within each survey year, samples of abalone were collected during each of 3 quarters (October – December, January – March, April – June), this design would also facilitate a test of the hypothesis that the intensity (and also prevalence) of infections increases during these 3 quarters (Spring, Summer, Autumn), as has been found for *Perkinsus* spp. in other hosts.

7.3.6. Clarification of the genetic and taxonomic status and geographic distribution of Perkinsus sp.-variant ITS

Identification of a *Perkinsus* sp. with a previously undescribed ITS-region gene sequence (*Perkinsus* sp.-variant ITS) suggests the presence of a new strain of *P. olseni* or alternatively, a previously undescribed species (Sections 4.3.2 and 4.4.2). Reece *et al.* (2001) identified 12 different genetic strains of *P. marinus* along the Atlantic and Gulf coasts of the USA, non-uniform geographic distribution of these strains and evidence that oysters could be infected by multiple genetic strains simultaneously. Based on the limited number of tissue samples from the 2005 survey on which PCR's and gene sequencing were done ($n = 31$, Section 4.2.1.3) and the origin of the samples (locations) both the relative frequency of *P. olseni* and *Perkinsus* sp.-variant ITS infections in abalone and the geographic distribution of *P. olseni* and the variant are poorly determined.

Further development of PCR methods to efficiently distinguish *P. olseni* and the variant is indicated. Provided that such methods were established prior to, or at the time of, future broad-scale surveys of *P. olseni* infections in abalone in NSW, this survey would provide clarification of the relative frequency and geographic distribution of *P. olseni* and the variant in abalone along the NSW coast. Moreover, differences could be compared between *P. olseni* and the variant in the intensity of infections, tissue destruction and host responses that occur. It is important to determine whether there is any difference in the virulence of the strains.

Given the lack of knowledge about intra-specific genetic variation in *P. olseni* across Australia, it would be valuable to compare samples of *P. olseni* and *Perkinsus* sp.-variant ITS with samples of *P. olseni* from a range of hosts from other states (S.A., Qld and W.A. in particular).

7.3.7. Confirmation of the absence of Perkinsus sp. in abalone on the majority of the far south coast of NSW

Whilst possible, it seems unlikely that *Perkinsus* sp. infections existed in abalone sampled from the stretches of coastline in 2002 and 2005 for which no infections were detected (Chapter 4). This

suggested that it is unlikely that *Perkinsus* sp. has been endemic and widely distributed along the coast of NSW since many years prior to the epizootics (Section 6.4.2). In order to dismiss the possibility that low-grade infections of *Perkinsus* sp. occur in organs (other than gill) and do not simultaneously exist in gill at these locations, testing for the presence of *Perkinsus* sp. using a method of greater sensitivity than histology is required.

As a component of future broad-scale surveys, application of a suitably sensitive testing methodology for the identification of *Perkinsus* sp., across multiple organs would provide further evidence for the presence/absence of *Perkinsus* sp. infections in abalone sampled from sections of the NSW coast for which infections were not detected during the 2005 survey. As discussed in Section 4.4.1 and Section 7.3.3, further development of real-time PCR assays accompanied by a formal study of the comparative sensitivity of real-time PCR, histology and Ray's test would be valuable. An established real-time PCR with sensitivity equivalent to Ray's test, but with increased specificity and application to tissue samples from a wider range of organs would facilitate fast and efficient testing for the presence of *Perkinsus* sp. in multiple tissue samples from multiple organs from multiple abalone sampled from survey sites.

7.3.8. Identification of alternative hosts for Perkinsus sp. in NSW

The limited number of alternative mollusc hosts sampled from sites and locations in NSW prevents the conclusion that *Perkinsus* sp. does not infect species other than blacklip abalone in NSW. Moreover, the involvement of intermediate hosts in the transmission of perkinsosis along the NSW coast cannot be discounted. Sampling of mollusc species other than abalone and subsequent testing for the presence of *Perkinsus* sp. infections, in conjunction with a repeat of the broad-scale survey of *Perkinsus* sp. infections in abalone, would directly address this uncertainty.

7.3.9. Investigating the possible link between reseeding experiments and transmission of Perkinsus sp. to sites on the south coast of NSW

As an adjunct to any future broad-scale survey of the prevalence of *Perkinsus* sp. infections in abalone along the NSW coast, it would be useful to include additional survey sites at and in the vicinity of sites at which juvenile abalone were released during the reseeding experiments during 1999 – 2001 (Tura Pt near Merimbula and several sites near Eden). This would facilitate testing the hypothesis that *Perkinsus* sp. infections exist in abalone at sites in close proximity to reseeding sites but do not exist at sites away from reseeding sites. This would provide additional evidence relating to the possibility that the releases of larval and juvenile abalone during reseeding experiments done in 1999 – 2001, were responsible for the active transmission of *Perkinsus* sp. into populations of abalone to the south of the southern "front" of infection at South Jervis Bay.

7.4. Conclusions

- A precautionary approach to the management of populations of abalone affected by *Perkinsus* sp. in NSW is warranted. Understanding of the epizootiolgy of perkinsosis is NSW and in other Australian jurisdictions is incomplete and there is potential for further impact of the disease on the commercial and recreational fishery concentrated on the south coast of NSW.

- Closures to fishing in areas where *Perkinsus* sp. is known to be present, accompanied by closures in buffer zones between infected areas and disease-free areas is one option for limiting the possibility of transmitting *Perkinsus* sp. from areas within which the parasite is known to occur into disease-free areas.

- Another approach to minimising the risk of *Perkinsus* sp. transmission concerns modification of existing fishing practices that involve a risk of transmission. "Standard Operating Procedures" for the decontamination of equipment (tools, boats, people and clothing, transport

containers) provides a means of minimising this risk. Such procedures may be voluntary (as at present) or may be a legal requirement for operation in the fishery.

- In the absence of closures preventing the harvesting of abalone potentially infected with *Perkinsus* sp., it is inevitable that abalone infected with *Perkinsus* sp. will be received by abalone processors. A review of current procedures used by processors and audit against best practice procedures is recommended.

- To counter the risk of transmitting *Perkinsus* sp. (and abalone viral ganglioneuritis), the distribution and use of abalone viscera as bait or burley was made illegal in NSW in December 2007.

- Given the absence of fishery-dependent data since the closures to commercial fishing between Port Stephens and Jervis Bay and limited fishery-independent survey sites, assessment of the current status of the severely depleted abalone stocks in this region is problematic. Further development of the structured fishing survey, first done in 2007, involving a limited number of commercial abalone divers fishing at specified "historically productive" drops between Port Stephens and Jervis Bay is recommended.

- Future monitoring of abundances of abalone at fishery-independent survey sites within this region, with possible expansion of the number of sites within Region 1 of the fishery, provides another means of monitoring abundance and recovery of stocks in this region of the fishery and is recommended.

- Whether abalone stocks in *Perkinsus* sp.-affected areas of Region 1 can sustain commercial harvesting has not yet been determined. In addition to controlling risks of transmission of *Perkinsus* sp., a future harvest strategy for this region will need to recognise the possibility that there may be future mortality events due to *Perkinsus* sp. in addition to mortality from future fishing.

- Given the incomplete understanding of the epizootiology of perkinsosis and associated mortality of abalone in NSW, high-priority monitoring and research projects were identified that would provide: information about any extension to the geographic range of *Perkinsus* sp. in NSW; the tissue and organ destruction associated with mortality; further understanding of the component factors (water temperature in particular) of cause; the identification of alternative hosts; seasonal cycles of transmission and progression of infections; and the establishment, in Australia, of fast, efficient real-time PCR assays for the identification of *P. olseni* (and variants and other *Perkinsus* spp.) infections in molluscs.

8. BENEFITS

The commercial and recreational fishing sectors in NSW will derive the majority of benefits from this project, given the focus of the project on the cause of observed mortalities of blacklip abalone in NSW during the 1990's, associated pathogenesis and epizootiology and management consequences and options. Nominally, the original application for funding to FRDC estimated flows of benefits to commercial and recreational sectors in NSW (64%), SA (15%), Tasmania (7%), Victoria (7%) and WA (7%). Benefits to these states derive from the presence of significant fisheries for abalone in these states and the identification of *P. olseni* or *Perkinsus* sp. in abalone populations in NSW, SA and WA. Although *Perkinsus* has not been identified in stocks of abalone in Victoria or Tasmania, benefits also accrue to these states due to the size and economic importance of their abalone fisheries and the potential for future infections of *Perkinsus* in these states.

Benefits to the commercial and recreational sectors in NSW derive from furthering the understanding of historical mortality events (Chapter 3), the recent geographic distribution, prevalence and intensity of *Perkinsus* sp. infections (Chapter 4), pathogenesis (Chapter 5) and epizootiology (Chapter 6) that inform management actions, and future research (Chapter 7). The extensive set of conclusions from this project, representing our improved knowledge of perkinsosis and associated mortalities in NSW is provided in Chapter 10. Minimising risk of transmission of *Perkinsus* sp. to populations of abalone currently unaffected by the parasite contributes to maintenance of productivity of these stocks and the subsequent economic benefits to fishers, local communities and the state.

Conclusions from this project concerning perkinsosis and mortality events of abalone in NSW and an extensive literature search on associated topics provide a reference for other states in examining the geographic distribution of *Perkinsus* infections in their stocks, pathogenesis and epizootiology. Conclusions relating to methodologies, the relative sensitivity of Ray's test, histology and PCR in particular, are also of fundamental importance for diagnosing *Perkinsus* infections in all states. Recommendations regarding future research priorities may also result in future benefits to fisheries in all states of Australia. For example, development and establishment of real-time PCR assays for the identification of *Perkinsus* spp. would provide benefits to all jurisdictions monitoring their stocks for *Perkinsus* infections and/or monitoring extensions in geographic range of infections.

9. PLANNED OUTCOMES

The objectives of this project concerned: documentation and compilation of historical evidence about the spread of *Perkinsus*-related mortality of abalone in NSW (Objective 1); describing the pathogenesis and making initial investigations of the epizootiology associated with documented mortality events, with particular reference to the role of *Perkinsus* (Objective 2); and to contribute to the development of strategies to manage populations of abalone that have, or might be affected by *Perkinsus*-related mortality and, in particular, to evaluate the need for further research Objective 3). Within a hierarchical structure, these general objectives were broken down into a second level of specific objectives (Chapter 2) and each of these objectives has been achieved within this project.

Historical evidence about mass mortalities of abalone in NSW was collected by interviews with divers and the timing and locations of these events was documented. Based on information from interviews with divers, fishery-dependent catch and effort data from the fishery and fishery-independent survey data, the depletion of abalone stocks prior to and since documented mortality events was estimated (Chapter 3). Information from these sources informed several important conclusions. Mass mortalities of blacklip abalone, observed by divers occurred during the year (+/- 1 year) in 1992 on the Central Coast, 1993 at Sydney-Wollongong, 1996 at Kiama, and in 2000 at Port Stephens. A sudden decline in abundance, unaccompanied by observations of morbidities or mortalities occurred at Jervis Bay during 2000 – 2002. Stocks of abalone were significantly depleted, due to fishing, by at least 63% prior to the documented mortality events in the 1990's; and stocks were subsequently depleted by approximately another 74% due to a combination of the documented mortality events during the 1990's and limited fishing. These estimates suggest that stocks may have been depleted to less than 10% of virgin abundance (Sections 3.5 and Chapter 10). These methods, analyses and conclusions fulfil Objective 1 of the project.

The broad-scale survey of the prevalence and intensity of *Perkinsus* infections in abalone completed in 2005 and related analyses and conclusions underpinned analyses concerning the pathogenesis and epizootiology presented in subsequent chapters. Understanding the relative sensitivity of diagnostic methods (Ray's test, histology, PCR and associated gene sequencing) informed the interpretation of survey results. *P. olseni* was one species of *Perkinsus* positively identified as infecting blacklip abalone in NSW. In addition, a previously unidentified strain of *P. olseni* or previously unidentified *Perkinsus* species (*Perkinsus* sp.-variant ITS) was also detected by PCR and subsequent gene sequencing. Prevalence and intensity of *Perkinsus* sp. infections in abalone were compared among locations, among sites within locations and with information from a previous survey done in 2002. The geographic distribution of *Perkinsus* sp. infections in abalone in 2005 represented an extension to the previously documented geographic range. Patterns in the prevalence and intensity of *Perkinsus* sp. infections in abalone among locations, among sites within locations and between 2002 and 2005 subsequently informed analyses concerning pathogenesis (Chapter 5), epizootiology (Chapter 6) and management and research options (Chapter 7).

The objective to describe the pathogenesis of perkinsosis associated with documented mortality events (a component of Objective 2) was addressed in Chapter 5. Infections of *Perkinsus* sp. detected by histology were generally systemic with intestine, gill, kidney, stomach and heart most frequently infected. Evidence of substantial tissue necrosis, organ damage and haemocyte activity occurred in abalone with high-intensity infections and provided direct evidence that *Perkinsus* sp. is pathogenic to blacklip abalone in NSW. Infiltration and aggregation of haemocytes provided evidence of host response to *Perkinsus* sp. and there was a positive relationship between infection intensity and haemocyte activity. The lack of encapsulation of *Perkinsus* sp. cells by haemocytes,

as described for *Perkinsus* sp. infections in blacklip abalone in SA, suggested a difference in virulence of *Perkinsus* sp. (or strain) and/or difference in the resistance of abalone in NSW compared to SA. Histology detected no pathogens other than *Perkinsus* sp. Infection intensity did not affect the length-weight condition of abalone sampled in 2005 (with Ray's test grade ≤ 3). No conclusions about the primary portal for entry of *Perkinsus* sp. into abalone were possible. However, evidence of gill infections in the majority of instances in which infections were identified by histology in a single organ was consistent with the gill as a portal of entry. These findings fulfil the component of Objective 2 concerning pathogenesis.

The objective to make initial investigations of the epizootiology of perkinsosis and associated with documented mortalities (a component of Objective 2) was addressed in Chapter 6. The importance of population variables including gender and size on prevalence and intensity of infections was examined. A small-scale survey of *Perkinsus* sp. infections in alternative mollusc hosts, at sites where *Perkinsus* sp. was prevalent in abalone, did not identify any alternative host species. Investigations were made of the likelihood of environmental variables (acting alone or as auxiliary factors operating with *Perkinsus* sp.) as the cause of documented mortality events. There were no environmental factors that were consistently associated by interviewed divers with mortality events that were consistent across the documented mortality events. The potential to examine associations between water quality and observed mortality events was limited by the spatial and temporal scales at which such data was available. The documented mortality events at specific locations in specific years were not consistently associated with increases, decreases, maximum or minimum values in any of the indices of water temperature examined. Mortality events were, however, associated with indices of water temperature in excess of 20°C and the greater intensity of infections identified in 2002 compared to 2005 corresponded with greater water temperature in 2002 compared to 2005. Based on the consistency of findings from this project and from other studies in Australia and internationally, with hypotheses concerning factors involved in the cause of documented mortality events, the likelihood of alternative hypotheses of cause being correct was graded "low", "medium" or "high". It was concluded highly likely that *Perkinsus* sp. was a necessary factor for the documented mortality events. The likelihood that water temperature (above some threshold) was an additional component of cause was also considered high. Whether or not another environmental factor(s) was also a component of cause was indeterminate. It was also considered likely, based on available evidence, that *Perkinsus* sp. has not been endemic and broadly distributed along the NSW coast for many years prior to the documented mortality events. The spatial and temporal pattern of documented mortalities and finding that Perkinsus sp. infections were greatest at the boundaries of the infected zone were consistent with a progression of *Perkinsus* sp. through naïve populations of abalone. In addition to passive transmission, multiple fishing-related activities were identified that could have translocated abalone infected with *Perkinsus* sp. and facilitated transmission of the parasite into populations of abalone that were disease-free. In addition to these possible mechanisms for active transmission, experiments involving the translocation of abalone from the wild to a breeding facility and the subsequent translocation of hatchery-reared juveniles and larvae from the facility to the wild between 1999 and 2001 could have inadvertently translocated abalone carrying *Perkinsus* sp. infections. Whereas the spatial and temporal pattern of documented mass mortality events and of *Perkinsus* sp. infections between "North Coast" and "South Jervis Bay" was consistent with the passive transmission of *Perkinsus* sp. among adjacent populations of abalone, possibly supplemented by active mechanisms of transmission, the isolated "outpost" of *Perkinsus* sp. infection in abalone at Merimbula is likely to have resulted from an active mechanism of transmission. The methods associated with these investigations and the findings outlined here, fulfil the component of Objective 2 concerning "initial investigations of epizootiology".

The objective to contribute to the development of strategies to manage populations of abalone that have, or might be affected by *Perkinsus*-related mortality and, in particular, to evaluate the need for further research was addressed in Chapter 7. The potential for further impact of the disease on the commercial and recreational fishery, if the range of *Perkinsus* sp. infections in abalone

expands on the south coast of NSW, was highlighted. Closures to fishing in areas where *Perkinsus* sp. is known to be present and/or the voluntary or mandatory adoption of "Standard Operating Procedures" for the decontamination of equipment (tools, boats, people and clothing, transport containers) would provide a means of minimising the risk of transmission of Perkinsus sp. into disease-free areas of the coast. In the absence of closures preventing the harvesting of abalone potentially infected with *Perkinsus* sp., it is inevitable that abalone infected with *Perkinsus* sp. will be received by abalone processors. A review of current procedures used by processors and audit against best practice procedures is recommended. The distribution and use of abalone viscera as bait or burley was made illegal in NSW in December 2007. Assessment of the current status of severely depleted abalone stocks in Region 1 of the fishery was also considered and the further development of structured fishing surveys and continued fishery-independent surveys recommended. Given the incomplete understanding of the epizootiology of perkinsosis and associated mortality of abalone in NSW, high-priority monitoring and research projects were identified that would provide: information about any extension to the geographic range of *Perkinsus* sp. in NSW; the tissue and organ destruction associated with mortality; further understanding of the component factors (water temperature in particular) of cause; the identification of alternative hosts; seasonal cycles of transmission and progression of infections; and the establishment, in Australia, of fast, efficient real-time PCR assays for the identification of *P. olseni* (and variants and other *Perkinsus* spp.) infections in molluscs. The discussion of alternative management options and future monitoring and research needs fulfils Objective 3 concerning management strategies and future research priorities.

10. CONCLUSIONS

Reported mortality events and declines in abundance of abalone in NSW (Chapter 3)

- Mass mortalities of blacklip abalone, observed by divers, occurred during the year (+/- 1 year): 1992 on the Central Coast, 1993 at Sydney-Wollongong, 1996 at Kiama and 2000 at Port Stephens. A sudden decline in abundance of abalone, unaccompanied by observations of morbidities or mortalities, occurred at Jervis Bay during 2000 – 2002.

- Stocks of abalone between Port Stephens and Jervis Bay were significantly depleted by at least 63%, due to fishing, prior to the documented mortality events in the 1990's.

- Stocks of abalone between Port Stephens and Jervis Bay were depleted by approximately 74% due to a combination of the documented mortality events during the 1990's and limited fishing.

- The estimated 63% depletion of stocks prior to, and 74% depletion since the documented mortality events, suggests that the stock of abalone between Port Stephens and Jervis Bay may have been depleted to less than 10% of virgin abundance.

- Given limited larval dispersal and localised recruitment in abalone populations, the recovery of stocks of blacklip abalone between Port Stephens and Jervis Bay is likely to be a slow process.

Identification and geographic distribution of the parasite *Perkinsus* in abalone in NSW (Chapter 4)

- Ray's test on samples of gill tissue was the most sensitive diagnostic method for the detection of *Perkinsus*. Histology (on multiple organs/tissues) detected only 33% of the infections detected by Ray's test. PCR detected only 54% of the infections detected by Ray's test.

- *P. olseni* was positively identified, by PCR and gene sequencing, as one species of the *Perkinsus* genus infecting blacklip abalone in NSW.

- Identification, by PCR and gene sequencing, of a *Perkinsus* sp. with a previously unknown ITS-region gene sequence (*Perkinsus* sp.-variant ITS) suggests the presence of a variant strain of *P. olseni,* or alternatively, a previously undescribed species.

- In 2005, *Perkinsus* sp. infections in abalone were identified at multiple survey sites, within multiple survey locations between Point Perpendicular (near Laurieton and within location "North Coast") on the north coast of NSW and St. Georges Head (within location "South Jervis Bay). *Perkinsus* sp. was also identified at a single isolated site (Merimbula, within location "Tathra-Eden") on the far south coast of NSW.

- *P. olseni* was positively identified at multiple survey sites within multiple survey locations between Point Perpendicular (within location "North Coast") and St. Georges Head (within location "South Jervis Bay").

- *Perkinsus* sp.-variant ITS was positively identified at multiple survey sites within multiple survey locations between the northern side of Crowdy Head (within location "Crowdy Head")

and St. Georges Head (location "South Jervis Bay") and at a single isolated site (Merimbula, within location "Tathra-Eden") on the far south coast of NSW

- Detection of *Perkinsus* sp. infections in abalone during 2005, at locations to the north and south of the previously documented range of *Perkinsus*, represents an extension in the documented geographic range of *Perkinsus* sp. infections in blacklip abalone in NSW. Actual extension of geographic range since 2002 cannot be concluded because the northern and southern boundaries of distribution identified in 2005 were outside the geographic range of the 2002 survey.

- Prevalence of *Perkinsus* sp. infections in abalone in 2005 differed significantly among locations and among sites within locations.

- Prevalence of infections during 2005 was greatest in populations of abalone at the northern and southern margins of the infected zone between locations "North Coast" and "South Jervis Bay" (excluding the "outpost" of infection at Merimbula). This is consistent with maximum prevalence occurring at the boundaries of the infected zone (locations "Port Stephens" and "South Jervis Bay") during 2002.

- Intensity of *Perkinsus* sp. infections in abalone (based on Ray's test grade) differed significantly among locations in 2005.

- Although there was no significant difference in the prevalence of *Perkinsus* sp. infections in abalone in 2005 compared to 2002, intensity of infections was significantly greater in 2002 (Ray's test grade: 1 – 3) compared to 2005 (Ray's test grade: 0.5 – 4).

- The greater intensity of *Perkinsus* sp. infections in 2002 compared to 2005 explains the better relative sensitivity of Ray's test and histology in 2002 compared to 2005. This is because the relative sensitivity of these diagnostic methods is better for high-grade infections (96% for Ray's grade ≥ 2 in 2005) than low-grade infections (8% for Ray's grade < 2).

Pathogenesis of *Perkinsus* sp. in abalone in NSW (Chapter 5)

- Infections of *Perkinsus* sp. (including a subset of cases for which *P. olseni* was positively identified by PCR and gene sequencing) in abalone that were identified by histology were generally systemic with intestine, gill, kidney, stomach and heart most frequently infected. Less frequently infected were epipodium, the digestive gland, oesophagus, muscle and haemolymph within the hemocoel. These observations were consistent between samples from 2002-03 and 2005.

- Substantial tissue and organ damage occurred in abalone with high-intensity infections. Necrotic tissue associated with *Perkinsus* sp. trophozoites and tomonts was observed in samples from 2002-03 and 2005. In the most severely infections (2002-03), disruption of the gut epithelium and infarction in the gills, suggested impairment to normal nutrient absorption and respiration (Callinan and Landos, 2006).

- Infiltration and aggregation of haemocytes provided evidence of host response to infections of Perkinsus sp. (including a subset of cases for which *P. olseni* was positively identified) and there was a positive relationship between infection intensity (Ray's test grade) and haemocyte activity.

- The evidence of substantial tissue necrosis, organ damage and haemocyte activity associated with *Perkinsus* sp. cells in samples from both 2002-03 and 2005 provides solid evidence that *Perkinsus* sp. (and specifically *P. olseni*) is pathogenic to blacklip abalone in NSW.

- Pathogenicity of *Perkinsus* sp.-variant ITS remains unresolved.

- The lack of encapsulation of *Perkinsus* sp. cells by haemocytes, as described for *P. olseni* infections in South Australian blacklip abalone (Lester, 1986), suggests a difference in the virulence of *Perkinsus* sp. (or strain) and/or a difference in the resistance of blacklip abalone in NSW compared to South Australia.

- No pathogens other than *Perkinsus* sp. (and *P. olseni*) were identified by histology in tissue samples from the 2005 survey. Nor were any pathogens other than *Perkinsus* sp. identified in samples from the 2003-03 NSDA survey or in the limited samples associated with mortality events.

- Infection intensity did not affect the length-weight condition of abalone sampled in 2005. Because extremely high-grade infections of *Perkinsus* sp. (Ray's grade ≥ 4) were absent from samples in 2005, this conclusion is restricted to abalone with infection intensity $\leq$ Ray's grade 3.

- No conclusions about the primary portal for entry of *Perkinsus* sp. into abalone are possible. However, the finding that gill was infected in 4 of the 6 instances that histology detected *Perkinsus* sp. in a single organ, is consistent with the gill being the primary portal of entry.

The epizootiology of perkinsosis and mass mortality of blacklip abalone in NSW (Chapter 6)

- Prevalence of *Perkinsus* sp. infections was gender-specific with infections more frequent in female (10.9%) than in male (5.0%) abalone in 2005. There was no gender-specific difference in infection intensity.

- Prevalence and intensity of *Perkinsus* sp. infections in abalone during 2005 was not dependent on the size (length) of abalone.

- There has been no decline in abundance of abalone at fishery-independent survey sites (monitored since 2005) adjacent to the southern "front" of infection (located at St George's Head within location South Jervis Bay in 2005).

- No alternative host species for *Perkinsus* sp. in NSW have yet been identified. No *Perkinsus* sp. infections were identified in 4 mollusc species sampled near Jervis Bay in 2005. Neither were any *Perkinsus* sp. infections identified by Gill (2003) at sites near Jervis Bay and Port Stephens.

- There were no environmental factors that were consistently associated by interviewed divers with mortality events that were consistent across the documented mass mortality events (identified in Chapter 2).

- The potential to examine associations between water quality and observed mortality events was limited. Available time-series of water quality data do not cover the spatial and temporal scales at which abalone mortalities and detection of *Perkinsus* sp. have been documented.

- The documented mortality events at specific locations in specific years were not consistently associated with increases, decreases, maximum or minimum values in any of the indices of

water temperature examined. Mortality events were, however, associated with indices of water temperature in excess of 20°C (20°C, 21°C or 22°C for the 3 indices considered).

- The greater intensity of *Perkinsus* sp. infections identified in 2002 compared to 2005 corresponds with indices of water temperature that were also greater in 2002 than in 2005, consistent with the hypothesis that progression of infection intensity is related to water temperature.

- Among various hypotheses concerning factors involved in the cause of documented mortalities:

  - the likelihood that *Perkinsus* sp. was a necessary factor was considered "High";
  - the likelihood that water temperature (above some threshold) was an additional component of cause was considered "High"
  - the likelihood that these 2 factors were sufficient was considered "Medium"
  - the likelihood that an additional environmental factor was also involved was considered "Medium".

- Alternative hypotheses concerning the historical distribution of *Perkinsus* sp. on the NSW coast were considered and, based on consistency of hypotheses with available information, the hypothesis that

  "*Perkinsus* sp. has been endemic along the coast of NSW since many years prior to the documented mass mortalities and identification of *Perkinsus* sp. in abalone (and that spatial and temporal variations in an environmental factor determined the spatial and temporal distribution of detectable *Perkinsus* sp., pathogenicity and subsequent mortalities)"

  was considered less likely to be correct than the hypothesis that

  "Perkinsus sp. has not been endemic to the entire coast of NSW for many years prior to the documented mass mortalities and identification of *Perkinsus* sp. in abalone (and that detection of *Perkinsus* sp. in abalone at a location at a point in time is closely associated with the arrival of *Perkinsus* sp.*)."*

- The spatial and temporal pattern of documented mortalities (Chapter 3) and finding that prevalence of *Perkinsus* sp. infections was greatest at the boundaries of the infected zone (excluding the "outpost" of infection at Merimbula; Chapter 4) are consistent with an epizootic propagating through naïve populations of abalone.

- Multiple fishing-related activities were identified that could have translocated abalone infected with *Perkinsus* sp. and facilitated transmission of *Perkinsus* sp. into populations of abalone that were previously disease-free. Activities that involved translocations at spatial scales of less than 100's of km included: harvesting of abalone by divers at individual drops; hanging of abalone over the side of the boat or pumping water over abalone in the boat during a day's fishing; hanging of catch during multi-day trips; and shucking of abalone and discard of viscera. Activities that involved translocation of abalone at greater spatial scales included: movement of divers, boats and equipment between locations; movement of catches to processors and distributors; and the distribution of abalone viscera as bait.

- Experiments involving the translocation of abalone from the wild to a breeding facility and the subsequent translocation of hatchery-reared juveniles and larvae from the facility to the wild between 1999 and 2001 could have inadvertently translocated abalone carrying *Perkinsus* sp. infections.

- The spatial and temporal pattern of documented mass mortality events and of *Perkinsus* sp. infections between "North Coast" and "South Jervis Bay" is consistent with the passive transmission of *Perkinsus* sp. among adjacent populations of abalone but possibly supplemented by active mechanisms of transmission.

- The isolated "outpost" of *Perkinsus* sp. infection in abalone at Merimbula is likely to have resulted from an active mechanism of transmission. Such an active mechanism could have involved a mobile marine species or bird acting as a vector, the fishing-related activities that involved translocation of abalone or abalone viscera over 100's of km, or the translocation/reseeding experiments that included the release, in 2001, of hatchery-reared juvenile abalone at Tura Pt (5 km to the north of the Merimbula site at which *Perkinsus* sp. was identified in 2005).

Further development – management and research options (Chapter 7)

- A precautionary approach to the management of populations of abalone affected by *Perkinsus* sp. in NSW is warranted. Understanding of the epizootiolgy of perkinsosis is NSW and in other Australian jurisdictions is incomplete and there is potential for further impact of the disease on the commercial and recreational fishery concentrated on the south coast of NSW.

- Closures to fishing in areas where *Perkinsus* sp. is known to be present, accompanied by closures in buffer zones between infected areas and disease-free areas is one option for limiting the possibility of transmitting *Perkinsus* sp. from areas within which the parasite is known to occur into disease-free areas.

- Another approach to minimising the risk of *Perkinsus* sp. transmission concerns modification of existing fishing practices that involve a risk of transmission. "Standard Operating Procedures" for the decontamination of equipment (tools, boats, people and clothing, transport containers) provides a means of minimising this risk. Such procedures may be voluntary (as at present) or may be a legal requirement for operation in the fishery.

- In the absence of closures preventing the harvesting of abalone potentially infected with *Perkinsus* sp., it is inevitable that abalone infected with *Perkinsus* sp. will be received by abalone processors. A review of current procedures used by processors and audit against best practice procedures is recommended.

- To counter the risk of transmitting *Perkinsus* sp. (and abalone viral ganglioneuritis), the distribution and use of abalone viscera as bait or burley was made illegal in NSW in December 2007.

- Given the absence of fishery-dependent data since the closures to commercial fishing between Port Stephens and Jervis Bay and limited fishery-independent survey sites, assessment of the current status of the severely depleted abalone stocks in this region is problematic. Further development of the structured fishing survey, first done in 2007, involving a limited number of commercial abalone divers fishing at specified "historically productive" drops between Port Stephens and Jervis Bay is recommended.

- Future monitoring of abundances of abalone at fishery-independent survey sites within this region, with possible expansion of the number of sites within Region 1 of the fishery, provides another means of monitoring abundance and recovery of stocks in this region of the fishery and is recommended.

- Whether abalone stocks in *Perkinsus* sp.-affected areas of Region 1 can sustain commercial harvesting has not yet been determined. In addition to controlling risks of transmission of *Perkinsus* sp., a future harvest strategy for this region will need to recognise the possibility that there may be future mortality events due to *Perkinsus* sp. in addition to mortality from future fishing.

- Given the incomplete understanding of the epizootiology of perkinsosis and associated mortality of abalone in NSW, high-priority monitoring and research projects were identified that would provide: information about any extension to the geographic range of *Perkinsus* sp. in NSW; the tissue and organ destruction associated with mortality; further understanding of the component factors (water temperature in particular) of cause; the identification of alternative hosts; seasonal cycles of transmission and progression of infections; and the establishment, in Australia, of fast, efficient real-time PCR assays for the identification of *Perkinsus* sp. (and variants and other *Perkinsus* spp.) infections in molluscs.

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12. APPENDICES

12.1. Appendix 1 – Intellectual Property

It is recommended that all outcomes from this project and potential associated intellectual property become public domain.

12.2. Appendix 2 – Staff

I&I NSW (formerly NSW DPI) project staff:

Dr Geoffrey Liggins	Principal Investigator	BSc (Hons) M.Comp PhD
Dr Judith Upston	Fisheries Technician / Scientific Officer	BSc (Hons) PhD
Pascal Geraghty	Casual Fisheries Technician	BSc (Hons)

Entities (and staff) contracted for specific components of project:

NSW DPI Regional Veterinary Laboratory, Wollongbar (now part of I&I NSW)
(contracted to perform histopathology)
Dr Paul Gill Veterinary Research Officer
Nick Stevens Casual Technical Assistant

Virginia Institute of Marine Science (OIE reference laboratory in USA)
(contracted to perform PCR assays for identification of *Perkinsus*)
Dr Kimberly Reece Associate Professor, School of Marine Science

The Ecology Lab Pty Ltd
(contracted to provide diving services to supplement DPI staff during broad-scale survey)
Dr Craig Blount Senior Environmental Scientist

AusVet Animal Health Services Pty Ltd
(contracted to provide review of report with emphasis on aspects of epizootiology)
David Kennedy Director, AusVet

12.3. Appendix 3 – Background notes and questions used during structured interviews of commercial and recreational abalone divers regarding their history of fishing in Region 1, observations of morbidity and mortality events, historical changes in abundance of abalone and environmental variables.

12.3.1. Interview questions – commercial abalone divers

Historical diver survey - abalone stocks in region 1 and *Perkinsus*-related mortality.

Background:

Abalone stocks in the region 1 (Port Stephens to Jervis Bay) have declined and there is presently very little information regarding the magnitude and cause(s) of the decline. The parasite, *Perkinsus olseni*, has been identified as a pathogen causing disease and eventual death of abalone and has been implicated as a possible cause of the large-scale mortality in NSW. Other environmental factors, which may stress or kill abalone, cannot be ruled out. The present FRDC project aims to compile and document the historical evidence about the spread of the *Perkinsus*-related mortality of abalone in NSW. An important component of the research is to obtain information regarding the decline in abalone stocks by interviewing past and long-term abalone divers. Ideally, we would want to know abundance of abalone (all size classes) on fished reefs, through time. Also we would like observations of sick/ dead abalone, spread of *Perkinsus*, habitat change, water quality, illegal harvesting, recreational catch. Realistically, we would hope to gain further understanding of the abalone population decline in region 1 by asking abalone divers a series of questions (see below). Further, we would ask divers to indicate on maps the locations of previously productive reef within region 1, and include estimates of past catch (kg per year) from these reefs.

Questions:

1. Were you an owner/ diver or nominated diver?

2. What year did you commence abalone diving (on a commercial basis) and what year did you stop diving for abalone? What was the main reason you stopped diving?

3. When you started abalone diving where was your home port? If you moved away from your initial home port and were still diving for abalone, then please specify the year you moved, the port you moved to, and reason for the move.

4. What were the main areas (abalone sub-zones) you fished in region1 up until present? For each of the main areas please estimate the year(s) you fished for abalone.

5. Do you recall specific years when you either commenced or stopped fishing one of your main areas? If so, please specify a reason for the change.

6. What proportion (%) of your total fishing days for each year was spent in region 1? Approximately how many fishing days each year?

7. What was the typical distance you would cover in a day's fishing? What was the minimum and maximum distance you would travel in one fishing day?

8. For each of the main areas you fished in region 1 and assuming a normal fishing day, estimate your average daily catch rate for each of the following year groupings:

1975-1979
1980-1984
1985-1989
1990-1994
1995-2000
2001-present

9. Assuming a normal fishing day in Region 1, estimate your:
best catch rate
worst catch rate

10. Specify exceptional years when abundance of legal size abalone in region 1 was either very good or poor?

11. For each of the main areas you fished in region 1 do you recall years when abundance of under-size abalone was either very good or poor?

12 (a). How would you describe the rate of change in abundance of abalone (legal size) in region 1, for each year fished, up until the present?

DRAW ON GRAPH (Separate graphs for different areas if necessary; include size limits & closures if they are recalled easily)

12 (b). For each of these years did you notice large changes in habitat, ocean temperature, water quality or other environmental factors?

DRAW ON GRAPH

12 (c). Can you recall exceptional environmental events (floods, storms, sand inundation) that lead to either an increase or decrease in abundance of abalone in an area? Describe your observations

12 (d). Were there specific areas you fished, where abalone abundance declined suddenly (within the space of months)?

13 (a). How would you describe the rate of change in illegal fishing in region 1, for each year fished, up until the present? Can you estimate the number of times you observed illegal fishing (indicated by shell dumps or direct observations of illegal fishers), for each year you fished?

DRAW ON GRAPH

13 (b). Are there particular years that were outstanding in terms of frequency and magnitude of illegal fishing? Specify years

14 (a). How would you describe the rate of change in frequency of recreational fishing for each year fished, up until present?

DRAW ON GRAPH

14 (b). Are there particular years that were outstanding in terms of frequency and magnitude of recreational abalone fishing in the main areas that you fished? Specify years

15 (a). What fishing methods did you use (include how abalone were transported on the boat) and did you employ a deckhand? If there was a change in fishing methods please specify the year(s) of change and provide details.

15 (b). What method did you use to measure legal size abalone (in water or on the boat)? Also what did you do with the under-size abalone?

15 (c). Did you hang your abalone? If so please provide details (when and where; weight of abalone hung).

16. Did you observe abalone dead, or with abnormal signs (e.g. lesions, lethargic), on reefs in region 1? If so please provide details and specify year, area and approximate number of individuals observed.

17. What processor did you supply your product to and was the product processed as live, frozen, canned meat? If this changed over time then please specify the year(s).

18. Did you notice abalone from R1 in poor condition or receive comments back from processor regarding condition of abalone? If so please provide details and year.

19. Did you send letters to the Fisheries department of Minister regarding declines in abalone stocks in region 1 or similar matters (can you supply us with a copy of the letter or specify the year the letter was sent)?

20. Do you have old logbooks or notes on your diving, which we could access?

21.Please specify on the maps provided your main abalone drops (i.e. those regularly fished). For each drop, specify your average daily catch and best ever-daily catch. Do you recall outstanding catches on spots dived by other abalone fishers?

MAPS

22. Further comments on abalone fishery, which may be relevant to decline of stocks and Perkinsus?

23. Have you dived as a recreational fisher in the recent past and noticed legal size abalone on reefs in region 1? If so then please specify the locations on the maps and for each location rate the abundance as:

Very good (average catch rate 100 kg/ hr)
Good (average catch rate 50 kg/ hr)
Average (average catch rate 25 kg/ hr)
Poor (average catch rate < 15 kg/ hr)

MAPS

24. Have you recently noticed undersize abalone on reefs? If so then please specify locations on the maps, and give some indication of numbers (1,000's; 100's; 10's of individuals)
MAPS

12.3.2. Interview questions – recreational abalone divers

Recreational Abalone Divers (long term divers who were mainly based in Region 1)

1. What areas (sub-zones) in Region1 (Port Stephens to Jervis Bay component) did you dive for abalone? Show on a map your main diving spots

2. How often did you dive for abalone and what years?

3. How many abalones did you collect on average per trip?

4. What method (scuba, hookah, snorkel) did you use to collect abalone?

5. Did you use a boat or shore dive? If both, then what was main method?

6. How would you describe the rate of change in abundance of abalone (legal size) in Region 1, for each year fished, up until the present?

DRAW ON GRAPH

7. Were there specific years when relative abundance of abalone had either noticeably increased or decreased? Prior to this change did you notice major changes in habitat, ocean temperature, water quality or other environmental factors?

8. How would you describe the rate of change in illegal fishing in Region 1, for each year fished, up until the present?

DRAW ON GRAPH

9. How would you describe the rate of change in frequency of commercial fishing for each year fished, up until present?

DRAW ON GRAPH

10. Did you observe abalone dead, or with abnormal signs (e.g. lesions, lethargic), on reefs in Region 1? If so please provide details and specify year, area and approximate number of individuals observed.

11. Did you send letters to the Fisheries department of Minister regarding declines in abalone stocks in Region 1 or similar matters (can you supply us with a copy of the letter or specify the year the letter was sent)?

12. Do you have notes on your diving, which we could access?

12.4. Appendix 4 – Laboratory reports concerning samples of abalone supplied to the NSW Agriculture / NSW Fisheries Regional Veterinary Laboratory associated with reported morbidity or mortality events and *Perkinsus*, 1992–1996.

12.4.1. WN92/1337

LABORATORY REPORT **WN92/1337**

NSW Agriculture
Regional Veterinary Laboratory
Wollongbar NSW 2477
Telephone : 066 240 261
Facsimile : 066 240 276

Date submitted : 25.5.92
Date received : 26.5.92
Interim reports: 1.6.92

R W COOK:AP

Owner NSW FISHERIES
TERRIGAL
Submitted by S FIELD, NSW FISHERIES

Subject: MORTALITY
Species: ABALONE
RLP Board MAITLAND

EXAMINATION	RESULTS AND REMARKS
HISTORY	Suspected: MORTALITY: Fish (ABALONE) age Unknown, sex Unknown. Samples taken from waters off the southern shore (rock platform) of Terrigal 'skillion' Terrigal. Substrate: rock (boulders). Depth of water: 3-5 m. Area inspected: approx 45 m of ocean floor. Other examples were seen to be lying on bottom shell down either dead or near death. The bottom was littered with a large number of empty shells. Fish activity appeared to be normal. Ocean temperature was warm.
HISTOPATHOLOGY	**Further histological sections from both specimens have been examined and protozoan organisms consistent with Perkinsus sp. have been identified associated with foci of inflammation in the gill stroma, ovary and around other viscera. Individual organisms are usually 12-17µm in diameter, with a single vacuole often containing round eosinophilic bodies. There are also discrete 15-27µm in diameter clusters of organisms containing individual organisms 5µm in diameter.** **Sample 1: (Male)** **Gills: Perkinsus-like organisms are associated with foci of inflammation in gill stroma. There is also mild to moderately increased cellularity of the gill stroma in areas free of organisms.** **Viscera: Foci of inflammation containing small numbers of protozoa are present between visceral organs.** **Sample 2: (Female)** **Gill: As for Sample 1** **Viscera: As for Sample 1** **Ovary: Extensive areas of cell lysis with serous exudation and small to moderate numbers of Perkinsus-like organisms.**
CONCLUSION	**Suspected Perkinsus sp. infection.** ***The contribution of this infection to the mortality problem is uncertain.*** ***Perkinsus olseni has been reported in greenlip abalone (Haliotis laevigata) in an area of die-back in South Australia.*** ***I will discuss with Dick Callinan his requirements for further preserved and fresh specimens to further investigate the significance of the suspected Perkinsus sp. infection.***

J G BOULTON
OFFICER IN CHARGE
4 September 2008

12.4.2. WN93/1341

Note that the statement in the histopathology section of this report that states "This appears to be a protozoan organism but in much larger clusters than *Perkinsus* sp. identified at Nelson Bay in May, 1992" is in error and should refer to "Terrigal 1992" (WN92/1337) not "Nelson Bay 1992".

LABORATORY REPORT **WN93/1341**

NSW Agriculture
Regional Veterinary Laboratory
Wollongbar NSW 2477

Telephone : 066 240 261
Facsimile : 066 240 276

Date submitted : 3.6.93

Date received : 4.6.93

Interim reports:

G C FRASER:AP

Owner NSW FISHERIES
LAKE ILLAWARRA

Subject: MORTALITY
Species: FISH

Submitted by MR RB CALLINAN
NSW FISHERIES, WOLLONGBAR NSW 2477

RLP Board NSW BUT OMITTED

EXAMINATION	RESULTS AND REMARKS
HISTORY	Suspected: MORTALITY. Fish (ABALONE breed). Age adult. Sex unknown. Abalone specimens collected 26/5/93 offshore from Coalcliff. Dead shell in area (4metres depth) possible result of kill.
HISTOPATHOLOGY	4 x abalone No 1 - Male: No significant lesions. No 2 - Male: Muscle near mouth: in a localised area of infiltration by mononuclear cells, there are about 15 clusters up to 60µm in diameter composed of 15µm in diameter spherical bodies containing in section about 5 bodies, 7µm in diameter. This appears to be a protozoan organism but in much larger clusters than Perkinsus sp. identified at Nelson Bay in May, 1992 (maximum cluster diameter was 28µm). Nos 3 and 4 - Males: No significant lesions.
CONCLUSION	Sample 2: Unidentified protozoan cysts in muscle (incidental finding).

J G BOULTON
OFFICER IN CHARGE

22 October 2007

FINAL REPORT W WINTER, DFO LAKE ILLAWARRA

12.4.3. WN95/3672

LABORATORY REPORT **WN95/3672**

NSW Agriculture
Regional Veterinary Laboratory
Wollongbar NSW 2477

Telephone : 066 261 261
Facsimile : 066 261 276

Date submitted : 13.11.95

Date received : 14.11.95

Interim reports:

P A GILL:AP

Owner: R CHICK, NSW FISHERIES, 202 NICHOLSON PDE, CRONULLA NSW 2230

Subject:

Species: FISH

Submitted by: ROWAN CHICK, NSW FISHERIES RESEARCH INSTITUTE, NSW FISHERIES, CRONULLA NSW 2230

RLP Board: MOSS VALE

EXAMINATION	RESULTS AND REMARKS
HISTORY	Fish (ABALONE breed). Age unknown. Sex unknown. Caught 10.11.95, kept in ice slurry since. Currently in 10% formalin on ice.
HISTOPATHOLOGY	Muscle: There are extensive areas of liquefactive necrosis with infiltrates of haemocytes. Large numbers of Perkinsus sp. in various stages are present within the lesions. Similar lesions are present in the mantle and some internal organs.
CONCLUSION	Perkinsosis.

JOHN BOULTON
OFFICER IN CHARGE

22 October 2007

FINAL REPORT R CHICK, NSW FISHERIES

12.4.4. WN96/1001

LABORATORY REPORT **WN96/1001**

NSW Fisheries
Regional Veterinary Laboratory
Wollongbar NSW 2477

Telephone : 066 261 261
Facsimile : 066 261 276

Date submitted : 4.4.96

Date received : 4.4.96

Interim reports:

R B CALLINAN:SGJ

Owner NSW FISHERIES
202 NICHOLSON PDE
CRONULLA NSW 2230

Subject: RESEARCH PROJECT

Species: FISH

Submitted by R CHICK
NSW FISHERIES, CRONULLA NSW 2230

EXAMINATION	RESULTS AND REMARKS
HISTORY	Single abalone P - three abalone F.
GROSS FINDINGS	No significant lesions seen.
HISTOPATHOLOGY	**Abalone submitted 4.4.96** Single abalone: Low grade to moderate Perkinsus sp infection in foot, gill, mantle, gut wall. Group of 3 abalone: Low grade to severe Perkinsus sp infection in foot, gill, mantle, gut wall, gonad, digestive gland. **Abalone submitted subsequently** 1 Storm Bay (1 abalone): Low grade to severe Perkinsus sp infection of gonad, gill and foot. 2 Calm Bay (1 abalone): Low grade Perkinsus sp infection of foot, gill, gut wall, gonad. 3 McLennan (2 abalone): Low grade to severe Perkinsus infection of foot, gill, mantle, gut wall, gonad. 4 Boat Harbour (3 abalone): No abnormality seen. 5 Luvs Bay (2 abalone): Low grade to moderate Perkinsus sp infection of foot, gill, gut wall, gonad, digestive gland. Perkinsus sp organisms were present singly or in groups, and often in considerable numbers. Most response ranged from nil viable to severe inflammation and necrosis. Localised oedematous areas, sometimes associated with Perkinsus sp, were present in foot of most abalone.
CONCLUSION	Perkinsosis. All abalone affected except those from Boat Harbour. No evidence of fungal infection.

12.4.5. WN96/1440

LABORATORY REPORT **WN96/1440**

NSW Fisheries
Regional Veterinary Laboratory
Wollongbar NSW 2477

Telephone : 066 261 261
Facsimile : 066 261 276

Date submitted : 22.5.96

Date received : 23.5.96

Interim reports:

R B CALLINAN:GH

Owner NSW FISHERIES/R CHICK
CRONULLA

Subject: ABALONE INVESTIGATION
Species: FISH

Submitted by L JIANG
NSW FISHERIES, WOLLONGBAR NSW 2477

EXAMINATION	RESULTS AND REMARKS
HISTORY	Suspected: ABALONE INVESTIGATION. Fish (ABALONE breed). Age unknown. Sex unknown. Perkinsus investigation. Specimens collected from various locations along NSW South Coast.
HISTOPATHOLOGY	Sections of foot, gill, mantle, gut digestive gland examined from 10 abalone from each site. A moderately severe Perkinsus sp. infection in foot, gill and gut connective tissue was seen in one abalone only, from the Kiama site. All other abalone were negative.
CONCLUSION	Perkinsiosis detected at Kiama only. *It may be worth examining abalone from these sites when water temperatures are higher. It would also be interesting to compare test sensitivities for histopathology, thioglycollate test on tissue and the haemolymph test. We would require live abalone for the latter two tests.*

R B CALLINAN
SPECIAL VETERINARY RESEARCH
OFFICER (FISH DISEASES)

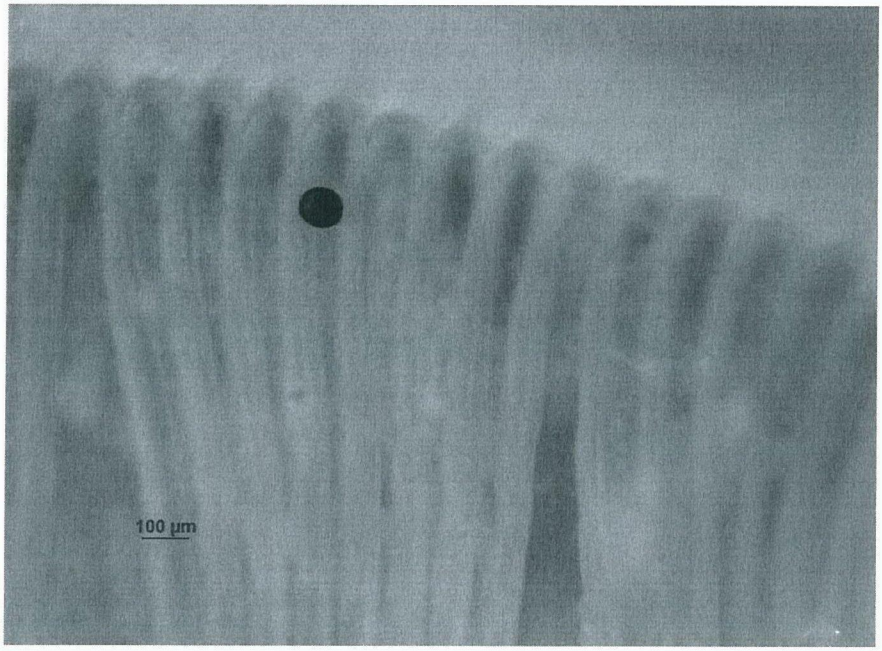
22 October 2007

FINAL REPORT R CHICK, FRI
SFO, NOWRA

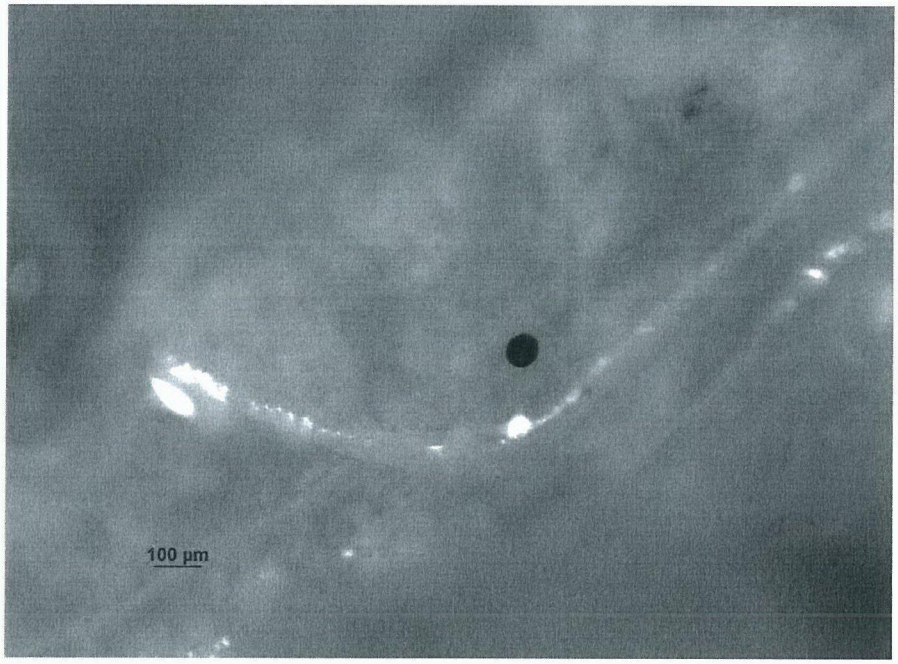
12.5. Appendix 5 – Images from diagnostic tests

12.5.1. Images of results from Ray' test

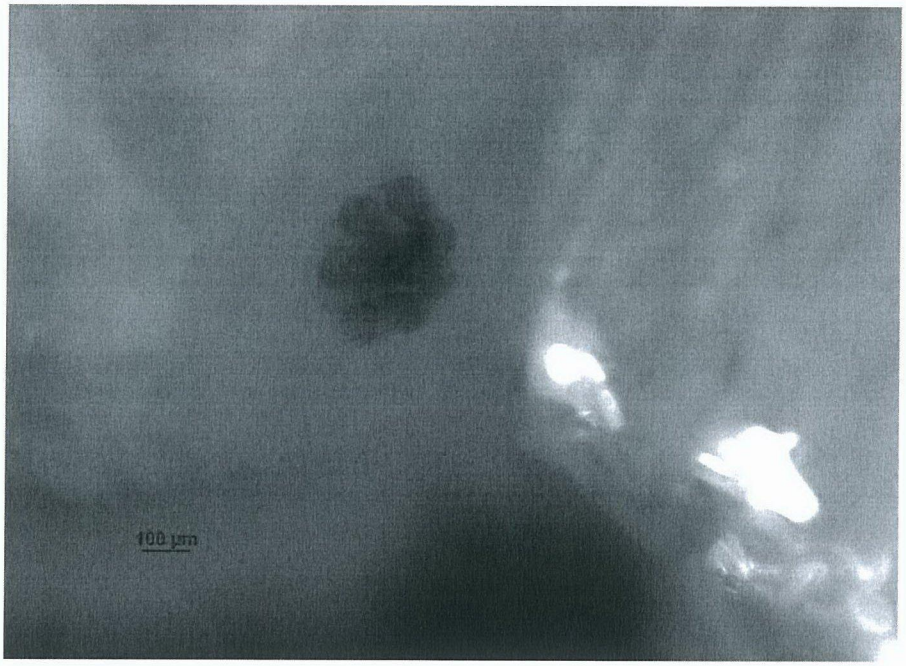
12.5.1.1. A single hypnospore on the surface of and between 2 lamellae of the gill.



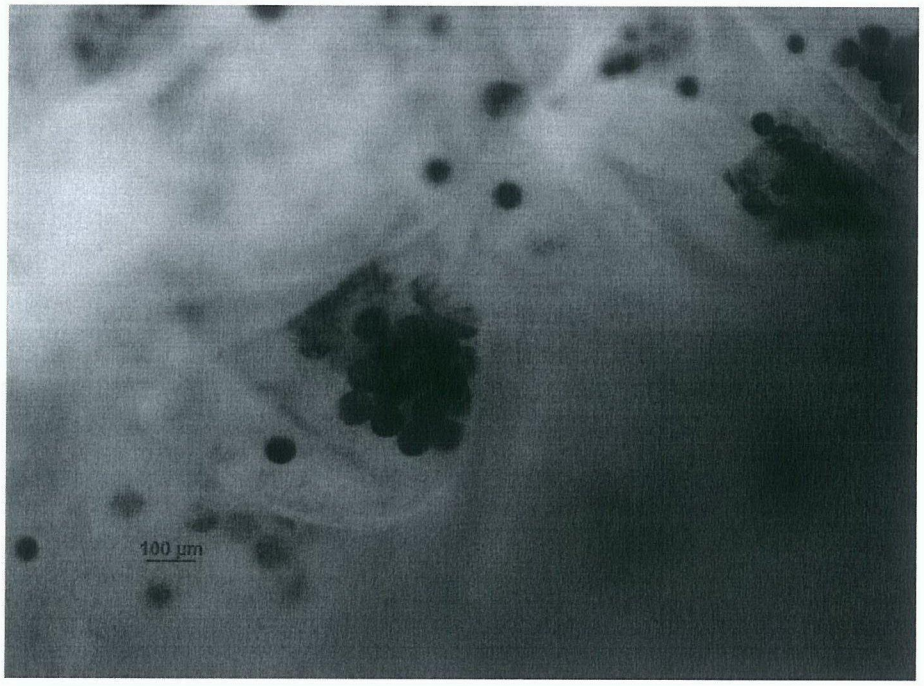
12.5.1.2. A single hypnospore within a lamella of a gill, low grade infection.



12.5.1.3. A cluster of hypnospores within and between gill lamellae.

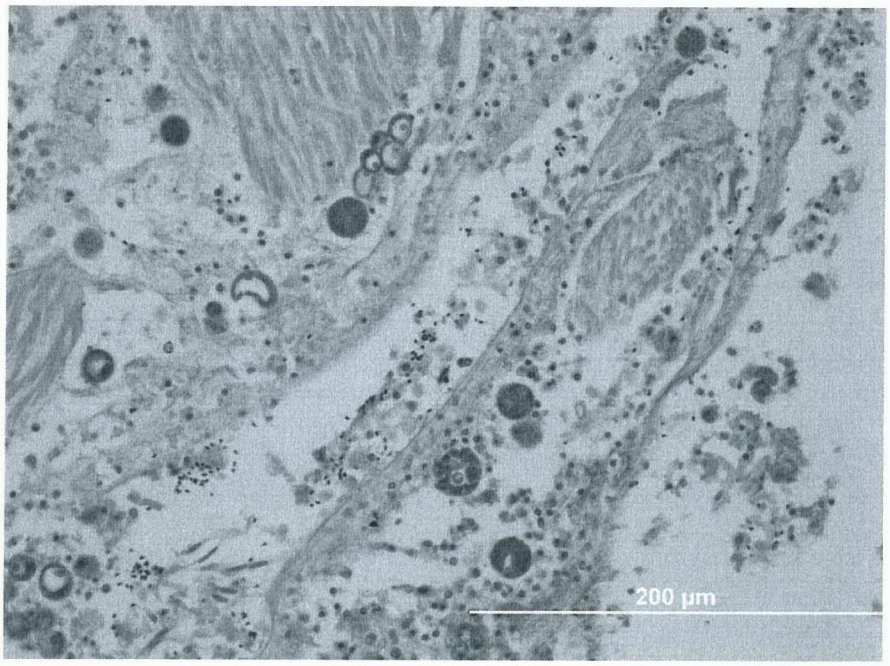


12.5.1.4. Hypnospores within and between gill lamellae, high-grade infection.

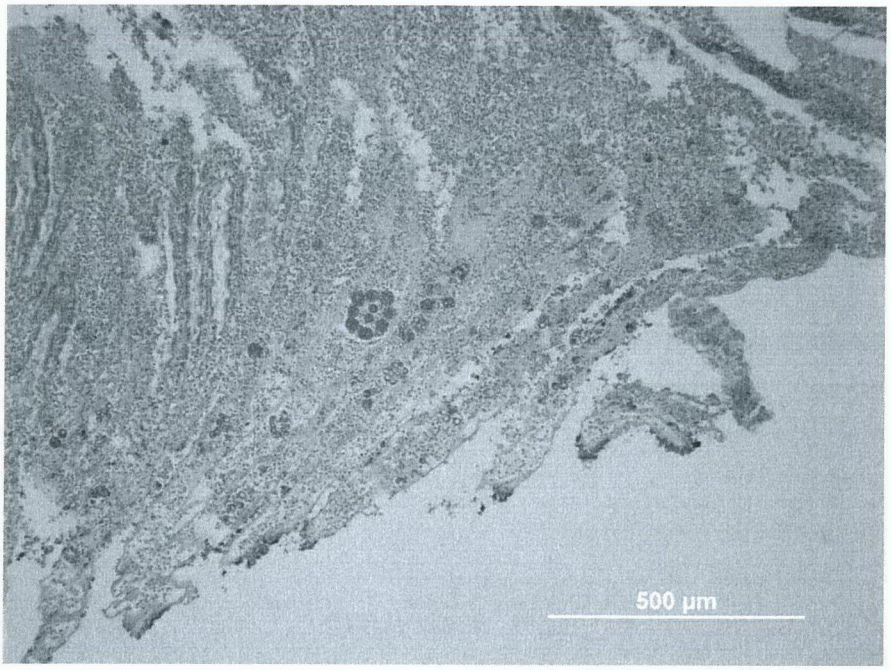


12.5.2. Images from histopathology (reprinted from Callinan and Landos, 2006)

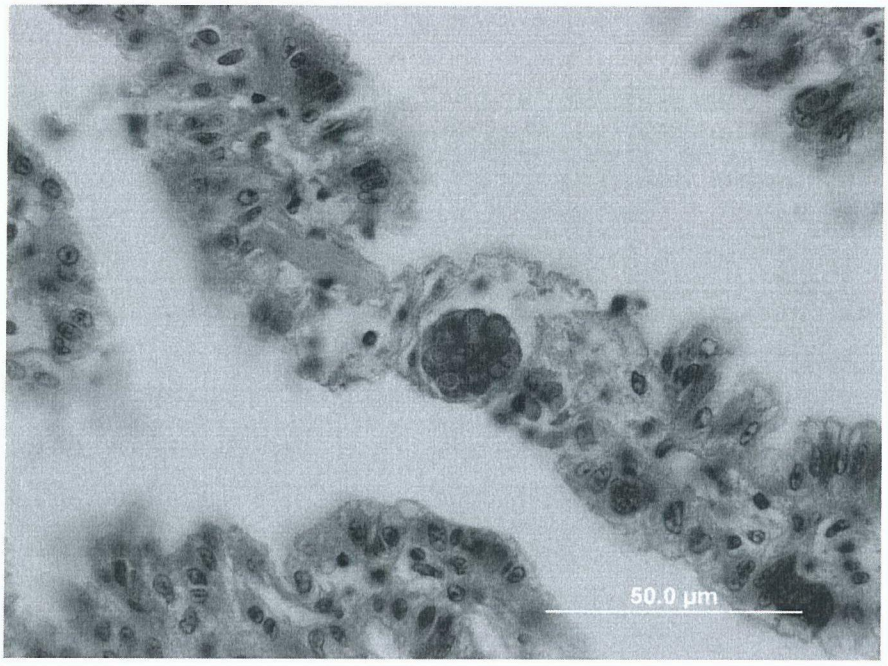
12.5.2.1. Perkinsus organisms surrounded by necrosis and tissue reaction.



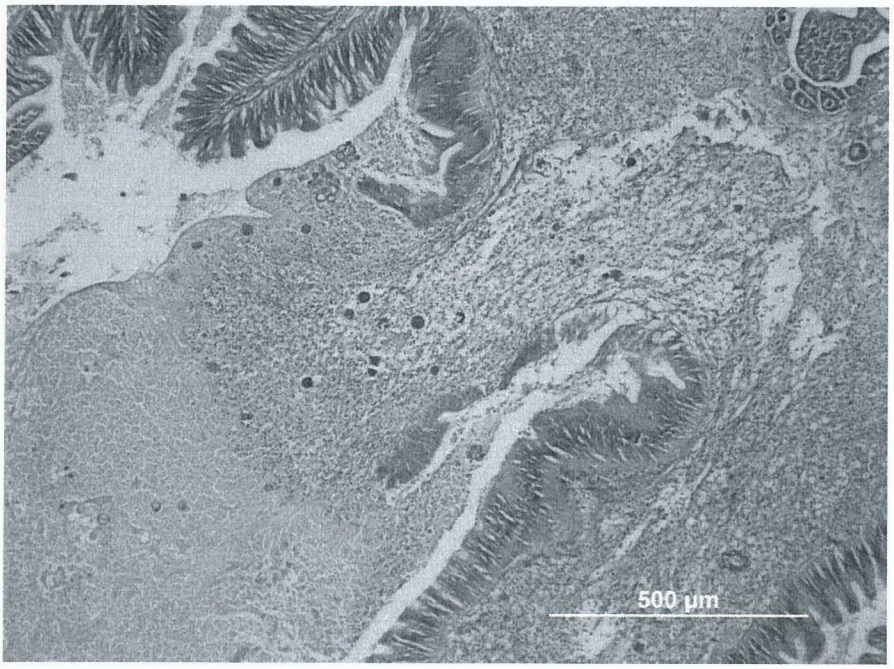
12.5.2.2. Multiple Perkinsus organisms blocking gill vessels, resulting in necrosis (paler areas with loss of detail).



12.5.2.3. Dividing Perkinsus parasite in gill vessel.



12.5.2.4. Perkinsus organisms and extensive inflammation under gut epithelium, leading to erosion of the epithelium and ulceration of the gut.



12.6. Appendix 6 – Suspected infections of *Perkinsus* sp. in abalone (positive results from Ray's test) sampled during broadscale surveys in 2002 and 2005.

Data is not shown for the 3 locations surveyed in the 2005 broad-scale survey that were not surveyed in 2002 (i.e., "North Coast", "South Bateman's Bay", "Tathra – Eden").
Column "M" refers to the month in which sampling occurred
Column "U" refers to the numbers of + test results from the 2002 survey for which archived tissue samples were unavailable for grading of infection intensity

Location	Site	2002										2005						
		M	N	Ray's test								M	N	Ray's test				
				+	0.5	1	2	3	4	5	U			+	0.5	1	2	3
Crowdy Head	Crowdy Nth	5	20	0	0	0	0	0	0	0	0	3	21	2	0	2	0	0
	Crowdy Sth	5	20	0	0	0	0	0	0	0	0	3	20	2	0	2	0	0
	Wallabi Point											3	20	0	0	0	0	0
	Red Head											3	20	0	0	0	0	0
	TOTAL		**40**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**		**81**	**4**	**0**	**4**	**0**	**0**
Forster/ Seal Rx	Lattitude Rock											3	21	0	0	0	0	0
	Forster	5	20	0	0	0	0	0	0	0	0		nil abs					
	Boomerang	5	20	0	0	0	0	0	0	0	0		nil abs					
	Seal Rocks (Lighthouse)	5	20	0	0	0	0	0	0	0	0	3	20	0	0	0	0	0
	Seal Rocks (Treachery)	5	20	0	0	0	0	0	0	0	0	3	20	0	0	0	0	0
	Yagan											3	20	0	0	0	0	0
	TOTAL		**80**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**		**81**	**0**	**0**	**0**	**0**	**0**
Port Stephens/ Broughton Is	Shag Rock (Broughton Is)	5	20	0	0	0	0	0	0	0	0		nil abs					
	Elizabeth Bay (Broughton Is)	5	20	0	0	0	0	0	0	0	0	2	20	4	3	1	0	0
	Tomaree											2	20	0	0	0	0	0
	South Fingal Is	2	21	5	0	0	1	1	1	0	2	2	20	1	1	0	0	0
	Boulder Bay	5	20	0	0	0	0	0	0	0	0	2	20	0	0	0	0	0
	Boat Harbour											2	20	1	1	0	0	0
	Fisherman's Bay	5	20	1	0	0	1	0	0	0	0		nil abs					
	TOTAL		**101**	**6**	**0**	**0**	**2**	**1**	**1**	**0**	**2**		**100**	**6**	**5**	**1**	**0**	**0**
Central Coast/ Sydney	Forresters	5	20	1	0	0	1	0	0	0	0	1	22	1	0	0	1	0
	Terrigal (Skillion)	5	20	0	0	0	0	0	0	0	0	1	21	0	0	0	0	0
	Newport	5	20	3	0	0	2	1	0	0	0	2	20	2	0	0	2	0
	Sand Shoes	4	20	0	0	0	0	0	0	0	0	1	21	0	0	0	0	0
	Curracarang	4	20	0	0	0	0	0	0	0	0	1	19	0	0	0	0	0
	TOTAL		**100**	**4**	**0**	**0**	**3**	**1**	**0**	**0**	**0**		**103**	**3**	**0**	**0**	**3**	**0**
Kiama	Bass Point	4	20	0	0	0	0	0	0	0	0	1	20	0	0	0	0	0
	The Farm											1	20	0	0	0	0	0
	Little Blowhole	4	20	1	0	0	1	0	0	0	0	1	20	0	0	0	0	0
	Obelisk	4	20	0	0	0	0	0	0	0	0	1	20	1	0	0	1	0
	Gerroa	4	19	1	0	0	1	0	0	0	0	1	20	1	1	0	0	0
	TOTAL		**79**	**2**	**0**	**0**	**2**	**0**	**0**	**0**	**0**		**100**	**2**	**1**	**0**	**1**	**0**
North Jervis Bay	Nth Beecroft											2	21	1	0	0	1	0
	Beecroft Head	5	20	4	0	0	1	1	1	1	0	2	20	7	2	3	2	0
	Targets	5	20	0	0	0	0	0	0	0	0	2	20	3	2	0	0	1
	Drum & Drum Sticks	5	20	0	0	0	0	0	0	0	0	2	20	0	0	0	0	0
	North Croc Head (Eyes)	5	20	9	0	1	3	1	3	1	0		nil abs					
	Croc Head	5	20	0	0	0	0	0	0	0	0	2	21	0	0	0	0	0
	TOTAL		**100**	**13**	**0**	**1**	**4**	**2**	**4**	**2**	**0**		**102**	**11**	**4**	**3**	**3**	**1**
South Jervis Bay	Sth Bowen Is	4	20	2	0	0	2	0	0	0	0	2	20	0	0	0	0	0
	Nth Stoney Creek	4	20	5	0	1	1	2	0	1	0	2	20	0	0	0	0	0
	Stoney Creek	4	20	9	0	0	6	0	0	2	1	2	20	1	0	1	0	0
	Steamers	5	20	3	0	0	1	2	0	0	0	2	20	0	0	0	0	0
	St Georges Head	4	20	0	0	0	0	0	0	0	0	2	20	11	2	5	4	0
	St Georges Sth											4	20	1	0	1	0	0
	TOTAL		**100**	**19**	**0**	**1**	**10**	**4**	**0**	**3**	**1**		**120**	**13**	**2**	**7**	**4**	**0**
Ulladulla	Sussex Inlet	4	20	0	0	0	0	0	0	0	0	1	20	0	0	0	0	0
	Bendalong	4	20	0	0	0	0	0	0	0	0	1	20	0	0	0	0	0
	Green Island	4	20	0	0	0	0	0	0	0	0	1	20	0	0	0	0	0
	Bannister Head	4	20	0	0	0	0	0	0	0	0	1	20	0	0	0	0	0
	Ulladulla (Lighthouse)	4	20	0	0	0	0	0	0	0	0	1	20	0	0	0	0	0
	TOTAL		**100**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**		**100**	**0**	**0**	**0**	**0**	**0**
Nth Bateman's Bay	Nuggan Point	4	20	0	0	0	0	0	0	0	0	3	20	0	0	0	0	0
	Brush Island	4	20	0	0	0	0	0	0	0	0	3	20	0	0	0	0	0
	Kioloa	4	20	0	0	0	0	0	0	0	0	3	20	0	0	0	0	0
	Snapper	4	20	0	0	0	0	0	0	0	0	3	20	0	0	0	0	0
	Pretty	4	20	0	0	0	0	0	0	0	0	3	20	0	0	0	0	0
	TOTAL		**100**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**		**100**	**0**	**0**	**0**	**0**	**0**

12.7. Appendix 7 – Standard operating procedures: decontamination for commercial abalone divers.

NSW DPI primefacts
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DECEMBER 2007 PRIMEFACT 714

Standard operating procedures: decontamination for commercial abalone divers

Aquatic Biosecurity and Risk Management

This Primefact presents some key hygiene protocols for abalone divers to help protect abalone stocks in NSW from disease. These standard operating procedures have been designed to minimise the disease translocation risk posed by abalone fishing.

Abalone viral ganglioneuritis

Abalone viral ganglioneuritis (AVG) is a herpes-like disease that was first detected in Australia in 2005 in southern Victoria, initially in farmed abalone and subsequently in wild stocks in 2006. The disease has resulted in extensive mortalities of greenlip and blacklip abalone in affected areas. Various management actions, including a fishing closure, have been unsuccessful in containing the disease, which currently spans almost 200 km of Victorian coastline.

Although similar to a virus found in farmed abalone overseas, AVG is not thought to be an introduced disease. Rather, it is considered to have been latent in native abalone populations, with disease expression triggered by accumulated stressors.

Figure 1. Healthy abalone (Photo: Mike Heasman)

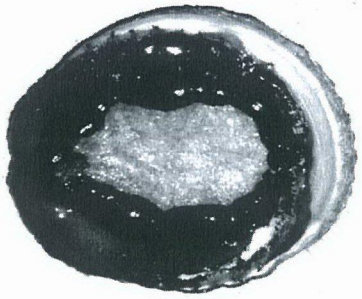

Figure 2. Diseased abalone displaying a symptom of AVG – curling of the foot (Photo: DPI Victoria)

There are no public health or food safety implications associated with this disease and it has not been reported in any other Australian states.

Symptoms

AVG affects the nervous system of abalone and symptoms include swollen mouthparts, curling of the foot resulting in exposure of shiny edges of the shell, difficulty adhering to surfaces, and lethargy, often causing death.

How is the virus spread?

Possible ways AVG is spread include direct contact between infected and healthy abalone, viral particles shed into the water column, and contact with infectious material (such as mucus) or contaminated equipment that has been used on diseased and then healthy abalone.

Detecting AVG

At present there are no diagnostic methods to detect this disease in asymptomatic abalone. However, Victoria DPI researchers, in conjunction with the Australian Animal Health Laboratory (AAHL), are working to develop a genetic-based

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diagnostic tool to detect this disease in abalone which may not be exhibiting obvious signs of disease or sickness.

Although there is no indication of this disease in NSW, NSW DPI is currently collecting and archiving abalone samples which, once a specific diagnostic tool is developed, will be used to investigate any presence of the disease agent in NSW.

Perkinsus

Perkinsosis is a disease that was first detected in NSW abalone stocks in 1992. It is an infection of molluscs caused by the genus of parasite *Perkinsus*, with *Perkinsus olseni* being the particular species that infects abalone. The disease has different symptoms from AVG. It causes abscesses, particularly in stressed abalone, which can result in mortality.

Perkinsus mortalities have contributed to stock decline, resulting in (ongoing) fishing closures. The impact of *Perkinsus* in NSW demonstrates the importance of safeguarding remaining abalone stocks from further disease incursions.

Hygiene protocols – standard operating procedures

To protect NSW abalone stocks from disease risks and the potential spread of diseases such as AVG and *Perkinsus*, NSW DPI wants to work with industry to develop standard operating procedures (SOPs) based on biosecurity best practice guidelines. The risk of aquatic disease outbreaks can be greatly reduced by applying appropriate biosecurity protocols through thorough hygiene practices. This SOP is designed to minimise the risk posed by activities associated with abalone fishing. Other SOPs are currently being developed to address risks associated with other sectors that deal with abalone, including abalone processors.

Decontamination

- Decontamination is important in preventing the transfer of disease.
- Decontamination procedures should be carried out when moving to a different fishing zone. To make decontamination more practical, divers should limit the spatial extent of their diving within each day.
- Decontamination procedures should be carried out when diving has ceased for the day. In particular, decontamination is recommended when diving in a different area from the previous day, e.g. diving in a northern area and then travelling south of the original diving location the next day.
- All equipment and persons that come into contact with abalone should be decontaminated.
- Effective decontamination generally requires a two step process, and involves a detergent wash followed by treatment with a disinfectant.
- Detergents remove gross organic matter, which impede the ability of disinfectants to inactivate pathogens. Disinfectants are then able to effectively inactivate or kill the pathogens.
- While diving, attention should be given to ensuring equipment, divers and decks are clean and routinely rinsed and kept free of any residue and organic matter such as seaweed, shell, sand etc. to promote effective decontamination.
- Although little is known specifically about AVG, similar viruses are inactivated by immersion in soapy water alone. However, as a precaution, and as part of best practice biosecurity decontamination measures, equipment should also be treated with a disinfectant.

Harvesting equipment decontamination

- All equipment that has come in contact with abalone, such as baskets, abalone irons, gloves, measures and catch bags, should be scrubbed clean and soaked in soapy water (which acts mainly as a detergent) for 30 minutes, then rinsed with fresh water.
- All such equipment should then be soaked in a 200 ppm effective chlorine solution prepared

Appendix - Preparation of a 200 ppm effective chlorine solution

Chlorine solution should be made up fresh for disinfection and not stored for any longer than 24 hours. Chlorine solution needs to be kept out of light and kept cool in order to minimise breakdown of chlorine.

To prepare a chlorine solution from granular pool chlorine:

1. Use only registered products containing 650 g/kg available chlorine present as calcium hypochlorite as the only active constituent as outlined in permit PER10371.

2. Dissolve 31 grams granular pool chlorine in 100 litres of water to produce a 200 ppm effective chlorine solution.

Note: adhere to all other instructions as indicated in permit PER10371 (valid until 27 Sept 2008).

Refer to the chemical product's Material Safety Data Sheets for instructions regarding the safe use of this chemical. This information should be available from the distributor when you purchase the product or by contacting the manufacturer.

from calcium hypochlorite e.g. pool chlorine (see Appendix) for an additional period of not less than 20 minutes, and then rinsed with fresh water.

- Throughout the diving operation, divers, equipment and decks should be rinsed to remove any organic material.
- Diving equipment that has not had direct contact with abalone, such as masks, buoyancy vests and regulators, can be washed with fresh water.
- After decontamination procedures are completed, allow equipment to dry in the sun before next use.

Vessel decontamination

- Throughout diving activity, decks and boating equipment should be regularly washed down (with fresh water if possible) and scrubbed, to remove all organic material such as sand, shell and seaweed.
- On returning to port, a thorough vessel cleaning should be undertaken. Cleaning should start from the highest point and work down to ensure all areas are cleaned. The deck and all parts of the boat that have had contact with abalone or abalone equipment should be thoroughly rinsed with fresh water, washed with disinfectant and rinsed again with fresh water.
- Once vessels are removed from the water the hull should be cleaned to remove any fouling organisms and organic material and should be hosed down with fresh water. Bungs should be removed for drainage and bilge areas rinsed with fresh water.
- Detergents specifically designed for cleaning boats should be used.

(Photo: John Matthews)

Decontamination of people and clothing

- Wherever possible, divers should rinse with fresh water between dives.
- Divers and any person who has handled abalone should wash their hands with soapy water.
- After diving, wetsuits should be washed both inside and out with a neoprene wash or with liquid soap or shampoo.
- Waterproof clothing should be rinsed with soapy water, rinsed with fresh water and dried in the sun.
- Gumboots and any footwear worn on the boat should be washed with soapy water, rinsed with fresh water and dried in the sun.
- Clothes should be washed with laundry detergent.

Decontamination of transport containers

- Containers which hold abalone should be thoroughly cleaned and disinfected after use.
- Remove any residue and organic material from containers.
- Soak containers with detergent, soak or wipe insides with disinfectant (a two minute contact time is recommended) and thoroughly rinse containers with fresh water. A thorough rinsing is necessary to ensure no harmful chemicals remain which can affect the next catch of abalone.

Decontamination procedures should be carried out away from the water's edge to prevent detergents and disinfectants from entering the marine environment.

Care should be taken to ensure that waste water from bilges is not discharged into the waterway.

Disposal of abalone shell, meat and gut

- Abalone should not be shucked at sea.
- Shell, meat and gut should not be discarded into marine waters and should instead be disposed with rubbish.

Reporting disease

1. If you observe any abnormality in abalone which may be indicative of disease, collect a sample and stop fishing.
2. Place the sample in a sealed plastic bag and add oxygen to the bag if possible. Keep the bag on ice and record the zone, specific location,

GPS coordinates, depth and symptoms the abalone is exhibiting.

3. Contact the NSW DPI Aquatic Biosecurity and Risk Management Unit on (02) 4982 1232 to provide details and to make arrangements for NSW DPI to collect the sample.

Help to protect abalone in NSW

- Decontaminate equipment and vessels during and after abalone diving.
- Plan your work to avoid or limit diving in different zones.
- Do not discard abalone or any part of abalone at sea.
- Divers are encouraged to not hang abalone outside the subzone in which they were collected, as this practice increases the risk of disease transmission from one location to another.
- Report anything you may notice in abalone that is unusual and could be an indication of disease to the NSW DPI Aquatic Biosecurity and Risk Management Unit on (02) 4982 1232.

ISSN 1832-6668

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