CONTROL OF *PERKINSUS* DISEASE IN ABALONE

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Final Report

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OBJECTIVES:

1. To determine the extent and nature of *Perkinsus* infection around Taylor Island.
2. To identify possible reservoir hosts
3. To produce an epidemiological model
4. To recommend appropriate management methods.

NON TECHNICAL SUMMARY:

OUTCOMES
The overall objective was to understand the epidemiology of *Perkinsus* disease in a specific well-known hotspot in South Australia. The hypothesis was that by removing a particular segment of the mollusc population, possibly an alternative reservoir host, the prevalence of the parasite in abalone would decrease. This project was to determine what mollusc species and if appropriate what ages should be removed. The results showed that there was no significant host in the area other than blacklip abalone, and transmission was not associated with juveniles but probably with rupturing abscesses on adult abalone. To test the conclusions, we suggest removing old abalone from a hotspot and measuring the rate of new infections over the following 4 months. Our demonstration of the parasite's low pathogenicity and its wide distribution in the world may lead to *P. olseni* being removed from the OIE list of notifiable diseases thereby removing a barrier to the national and international movement of live and chilled Australian abalone.

NON-TECHNICAL SUMMARY

Perkinsus olseni is a protozoan parasite that infects abalone species. Infections in blacklip and greenlip are often visible as brown nodules 0.5 to 8 mm in diameter on the upper part of the foot, the mantle or as internal pustules. These reduce the market value. The infection was thought to eventually kill the host abalone, or under conditions of cold temperature (below 15^{o}C), the abalone may successfully combat the infection. A very similar parasite has been found in South Australia in several species of bivalves.

The overall objective was to understand the epidemiology of *Perkinsus* disease in a specific well-known hotspot in South Australia. The hypothesis was that by removing a particular segment of the mollusc population, possibly a reservoir alternative host, the prevalence of the parasite would decrease.

Our molecular systematics demonstrated that the *Perkinsus* from Australian abalone and those from Australasian bivalves all belonged to the same species, *Perkinsus olseni*. This was shown to be a senior synonym for *Perkinsus atlanticus*. Thus the parasite is widely

distributed across the old world (Australasia, Korea, Japan, Portugal, Spain). As a result it is possible the Office International des Epizooties will remove it from their list of notifiable diseases making one less barrier to the export of Australian molluscs.

Survey data from the experimental site and adjacent areas showed that there was no significant host in the area other than blacklip abalone. 420 individuals representing 38 species of mollusc were examined. All but one of the 29 infections found were in blacklip abalone *Haliotis rubra*. The only exception was an infection in *H. roei*. Thus blacklip abalone was the primary host at the experimental site.

The parasite did not kill abalone in the laboratory, nor as far as we could tell in the field. Indeed the protozoan appears to be largely a commensal until encapsulated by the host and killed or ejected. The abscess formed during the encapsulation becomes surrounded by abalone pigment, hence the yellow/brown appearance. We hypothesise that transmission is not associated with abalone death but with rupturing abscesses on adult abalone which release zoospores into the environment to infect other abalone in the area. An alternate hypothesis is that infected abalone are eaten by fish, the parasites pass through the gut and form zoospores on being ejected in the faeces. The model now needs to be modified to delete parasite-associated mortality, to incorporate data on zoospore production through abscess formation, and to evaluate the role of fish predation.

The reason for the hotspots of infection in South Australia may relate to localities where abalone are occasionally stressed. This could be from sudden changes in temperature or from temporary food shortage (absence of floating weed from weed die-off or temporary change in currents). The stress on the abalone apparently allows the parasite to replicate before being contained by the abalone's immune response.

We conclude that a reduction in the number of animals with abscesses should result in a drop in the prevalence. The outcome recommends that old abalone be removed from the hotspot and the rate of new infections over the following 3 months be measured, possibly together with changes in the abundance of zoospores. The prevalence of the disease in the resident animals should decrease over the next several years making the area more valuable to the abalone industry.

KEYWORDS: abalone, disease, Perkinsus

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Parts of the field work in SA and the molecular study at the University of Queensland (UQ) were carried out by Dr Sarah Kleeman. Further molecular assistance was provide by Dr Steve Barker, UQ, and his group, particularly Dr Anna Murrell. Dr Hamish McCallum (UQ) advised on the epidemiological model. Mrs Thora Whitehead kindly

identified the molluscs from Grindal Island, SA, and Dr John Healy (UQ) molluscs from NSW.

BACKGROUND

Perkinsus olseni is a protozoan parasite that infects abalone species (Lester and Davis, 1981) (Fig. 1). Infections in blacklip and greenlip are often visible as brown nodules 0.5 to 8 mm in diameter on the upper part of the foot, the mantle or as internal pustules. These reduce the market value. The infection was thought to eventually kill the host abalone, or under conditions of cold temperature (below 15^{o}C), the abalone may be able to successfully combat the infection. A very similar parasite had been found in South Australia in several species of bivalves.

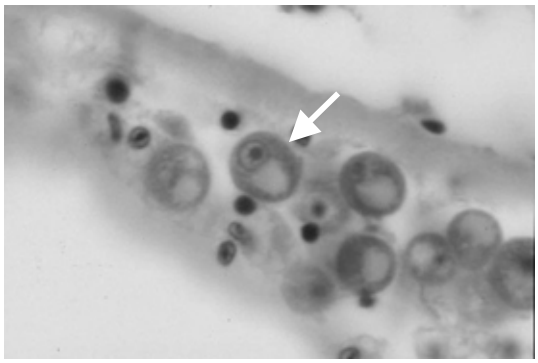

FIG. 1 Cells of *Perkinsus olseni* in haemolymph of *Haliotis laevigata* gill.

In abalone, the parasite occurs as a feeding stage or 'trophozoite' usually in the 'haemolymph' (blood) (Fig. 1). Trophozoites grow and eventually subdivide into 8, 16 or 32 daughter trophozoites (Perkins, 1966; Goggin and Lester, 1995; Sunila et al., 2001). These in turn grow and subdivide so that many clumps of trophozoites may be circulating in the haemolymph. When the abalone dies, fully developed trophozoites swell into 'hypnospores', the next stage in the life cycle, apparently in part because the environment is anaerobic. This may take a few hours to a few days. Small trophozoites and dividing forms die. If the hypnospores then come into contact with seawater, probably as the abalone tissue disintegrates, each swollen cell subdivides into several hundred minute zoospores which are released into the seawater through a specially formed 'discharge tube'. This takes a further 3 to 4 days. The zoospores infect a mollusc by penetrating through the epithelium of the skin or gill and start growing as trophozoites.

Healthy blacklip and greenlip abalone respond strongly to the presence of the parasites and often form an abscess around them, sealing them off from the body. This is the pustule seen by processors. Hypnospore formation can occur within these pustules and as the cells swell they are pushed out of the abscess onto the surface of the abalone.

Endemic foci of *Perkinsus* disease in abalone have been known since at least 1972 in South Australia (Lester and Davis, 1981), in several locations especially in the central and western zones. Periodic increases in prevalence have resulted in restrictions and/or closures to minimise the effect on the market (Shepherd et al., 1999).

Perkinsus parasites were recognised in abalone along the NSW coast in 1992. They were regarded as a contributory cause of death in recent abalone mortalities and an independent survey for infections was underway during this project (Gill, 2003; D. Worthington, NSW Fisheries, personal communication).

In an earlier FRDC project, Lester (1994) recovered *Perkinsus* sp. from four species of abalone and 5 species of bivalves in South Australia. These were *Haliotis rubra, H. laevigata, H. cyclobates, H. scalaris, Barbatia pistachia, Katelysia rhytiphora, Pinna bicolor, Chlamys bifrons,* and *Cleidotheidothaerus* sp. In Queensland, *Perkinsus* sp. was common in the bivalves *Anadara trapezia* and *Chama pacificus*.

Goggin (1994) showed that the Internal Transcribed Spacer regions (ITS1 and ITS2) of the ribosomal DNA gene in *Perkinsus* from South Australian abalone were virtually identical to that from *Perkinsus* in bivalves suggesting only one species of *Perkinsus* was present. Along the east coast of North America, Kotob et al. (1999) and Coss et al. (2001) found that the Non-Transcribed Spacer region (NTS) sequences were more useful to differentiate *Perkinsus marinus* from *P. chesapeaki* (and a synonym *P. andrewsi*) than the ITS regions. This suggested that the NTS sequences of Australian *Perkinsus* may reveal cryptic species not so far detected

The overall objective was to develop a method to control *Perkinsus olseni*. This was seen to be a two stage process with first the detection of the infection in hosts and understanding of its critical environmental requirements in one habitat. The second stage was a subsequent project to establish a control procedure and implement it.

Taylor Island was chosen as the experimental site. It is an uninhabited island 20 km southeast of Port Lincoln in a relatively sheltered site (Fig. 2). The abalone there are at least 2 km from other abalone habitat, thus forming a relatively isolated and discrete group, and they have a long history of *Perkinsus* infection particularly those on the west side.

The project developed in three parts, 1) the accurate diagnosis of infection, 2) survey data on host and parasite abundance together with field experiments and laboratory observations to form, 3) an epidemiological model from which a management plan could be derived.

NEED

Disease, particularly *Perkinsus* disease, was seen as the fourth major risk to the sustainability of the abalone fishery in South Australia (Anon., 2003). Other risks identified included illegal catch, collection of broodstock for aquaculture and stock enhancement projects.

Perkinsus disease causes ongoing losses to the industry, losses that are expected to increase with global warming unless some action is taken to curb the disease. The project was to explore one approach that may be feasible to control *Perkinsus* and thus reduce its impact on the industry.

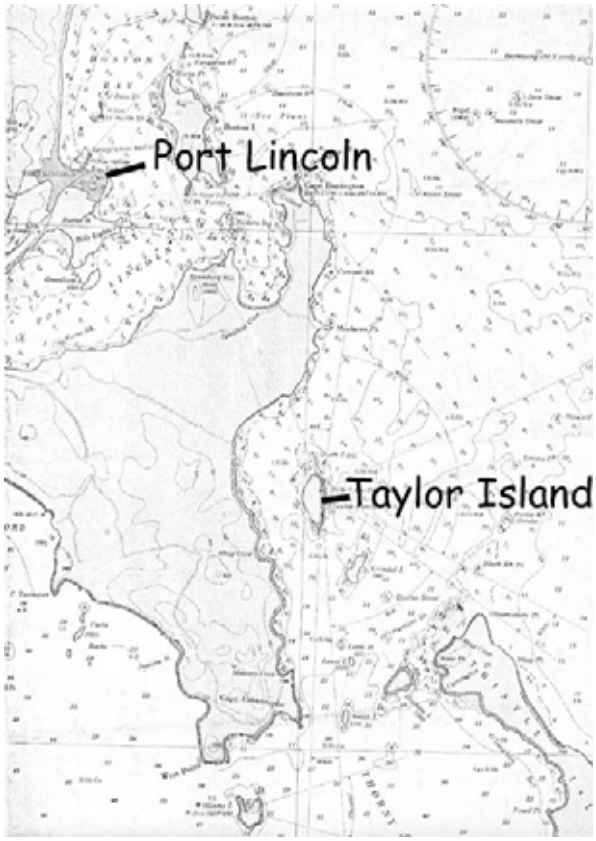

FIG. 2. Map of part of South Australia showing Port Lincoln and Taylor Island (34°53'S 136°00'E) .

OBJECTIVES

1. To determine the extent and nature of *Perkinsus* infection around Taylor Island.
2. To identify possible reservoir hosts.
3. To produce an epidemiological model.
4. To recommend appropriate management methods.

OBJECTIVE 1: TO DETERMINE THE EXTENT AND NATURE OF *PERKINSUS* INFECTION AROUND TAYLOR ISLAND.

1.1 ACCURATE DIAGNOSIS OF INFECTION

As stated above, *Perkinsus* sp. had been detected in many molluscs in Australia and although several *Perkinsus* species are recognised worldwide, there was little evidence that more than one species occurred in Australia. In North America, Kotob *et al*. (1999) and Coss *et al*. (2001) found that the Non-Transcribed Spacer region (NTS) sequences were useful to differentiate *Perkinsus* species. We therefore obtained NTS sequences of Australian *Perkinsus* to differentiate Australian species.

Methods

Mollusc tissues from field samples were incubated in thioglycollate medium at room temperature (approx. 22°C) for 5 to 10 days. *Perkinsus* cells were collected and frozen in

liquid nitrogen for later DNA extraction. The nontranscribed spacer (NTS) of the ribosomal RNA gene of the *Perkinsus* parasites was amplified by PCR using primers from Herran *et al*.(200) and sequenced. Sequences were obtained from *Perkinsus* parasites from blacklip and greenlip abalone, from Taylor Island and the immediate vicinity, and from bivalves from Moreton Bay, Queensland, and Auckland, New Zealand.

Results/Discussion

The NTS sequences from all isolates were at least 95% identical. The least similarity, 95.6%, was between a blacklip from Taylor Island and a greenlip from Taylors Landing 4 km away. There was more variability within the abalone samples than between the abalone and those from bivalves in Queensland and New Zealand. All isolates were at least 20% different from *Perkinsus marinus* and *P. andrewsi*.

The similarity of the Australasian *Perkinsus* sequences and the large differences between them and the sequences from North America strongly suggest that the *Perkinsus* infections we were working with in South Australia all belong to the same species, *P. olseni*. For other details see Murrell *et al*. 2002 (Appendix 3).

1.2 SURVEY DATA TO MONITOR PREVALENCE

In 8 of the 10 visits to the experimental site, blacklip were collected to obtain *Perkinsus* for the taxonomic study above, and to estimate the prevalence of the parasite for the population model. Other molluscs were also collected to determine the prevalence of *Perkinsus* in alternative or reservoir hosts.

Methods

The experimental site at Taylor Island was near the middle of the eastern shore at northern edge of the small bay. Molluscs were collected using SCUBA, hookah and snorkel, initially along two transects marked by a weighted 30 m rope set at right angles to the island (Fig. 3). Both transects ran east into water about 7 metres deep. Additional blacklip abalone were collected from Site 3, a 10 sq.m. area 5 m. deep immediately north of the cove and in between the first two sites. Later transects and quadrats were in the same area though not on exactly the same transect lines.

Molluscs were dissected at the Lincoln Marine Science Centre. Representative pieces of tissue, always including gill, were incubated in thioglycollate medium. Some duplicate pieces were fixed in Davidson's fixative for histology.

The thioglycollate samples were incubated at room temperature (approx. 18-22^{o}C) and were examined for *Perkinsus* 5 to 10 days later using the Ray test (Ray, 1966). Prevalence was estimated from the results of the Ray test as this is generally considered more reliable than histology (Goggin and Lester, 1995; McLaughlin and Faisal, 1999).

Results

Perkinsus olseni was present at a high level in most of the samples of wild blacklip from the experimental site (Table 1). Detailed data for two 1 sq.m. quadrats used on 10 May, 2001, are given in Appendix 4.

Discussion

The parasite was present throughout the year. Its estimated prevalence fluctuated between 19 and 68%, possibly in part due to variations within the site, variations between the years and the error introduced by small sample sizes. In previous work (Lester, 1994), large seasonal differences have been observed, high levels being found in the late summer. A further discussion of the possible causes is given below (Section 3.4).

TABLE 1. Prevalence of *Perkinsus olseni* in blacklip *Haliotis rubra* at the experimental site (north edge of small bay on east side of Taylor Island).

Date	No. examined	No. infected	(No. with abscesses)	Prevalence
19 June, 2000	42	11	(7)	26%
17 January, 2001	16	8	(4)	50%
13 February, 2001	30	17	(14)	56%
10 May, 2001	23	13	(5)	57%
30 January, 2002	16	3	(1)	19%
13 May, 2002	10	3	(0)	30%
24 January, 2003	16	10	(1)	63%
10 April, 2003	40	27	(4)	68%
Total	256	93	(36)	36%

OBJECTIVE 2: TO IDENTIFY POSSIBLE RESERVOIR HOSTS

The results of earlier studies (Goggin and Lester, 1987), and confirmed by Section 1.1 above, indicated that *Perkinsus olseni* is found in a range of molluscs. We therefore sought to determine what other molluscs were present at the experimental site and whether they were infected with *Perkinsus*.

Methods

Intensive searches for molluscs were conducted using SCUBA and hookah at the experimental site. An additional sample (May 2002) was taken from a nearby island, Grindal Island, 3 km SSE from Taylor Island. Pieces of molluscs including gill, or the whole mollusc for small species, were placed into thioglycollate and examined for *Perkinsus* using the Ray method.

Results

The only genus infected was *Haliotis*, and all but one were *H. rubra*, the blacklip abalone (Table. 2). In April, 2003, an hour-long underwater search in the seagrass adjoining the experimental site failed to locate any bivalves or other molluscs. A full list of species examined is given in Table 3.

TABLE 2. Numbers of molluscs examined for *Perkinsus* at Taylor and Grindal Islands.

Date	no. species examined	no. individuals examined	no. species infected
19 June, 2000	14	97	1
13 February, 2001	19	201	1
10 May, 2001	5	91	1
13 May, 2002	21	173	1

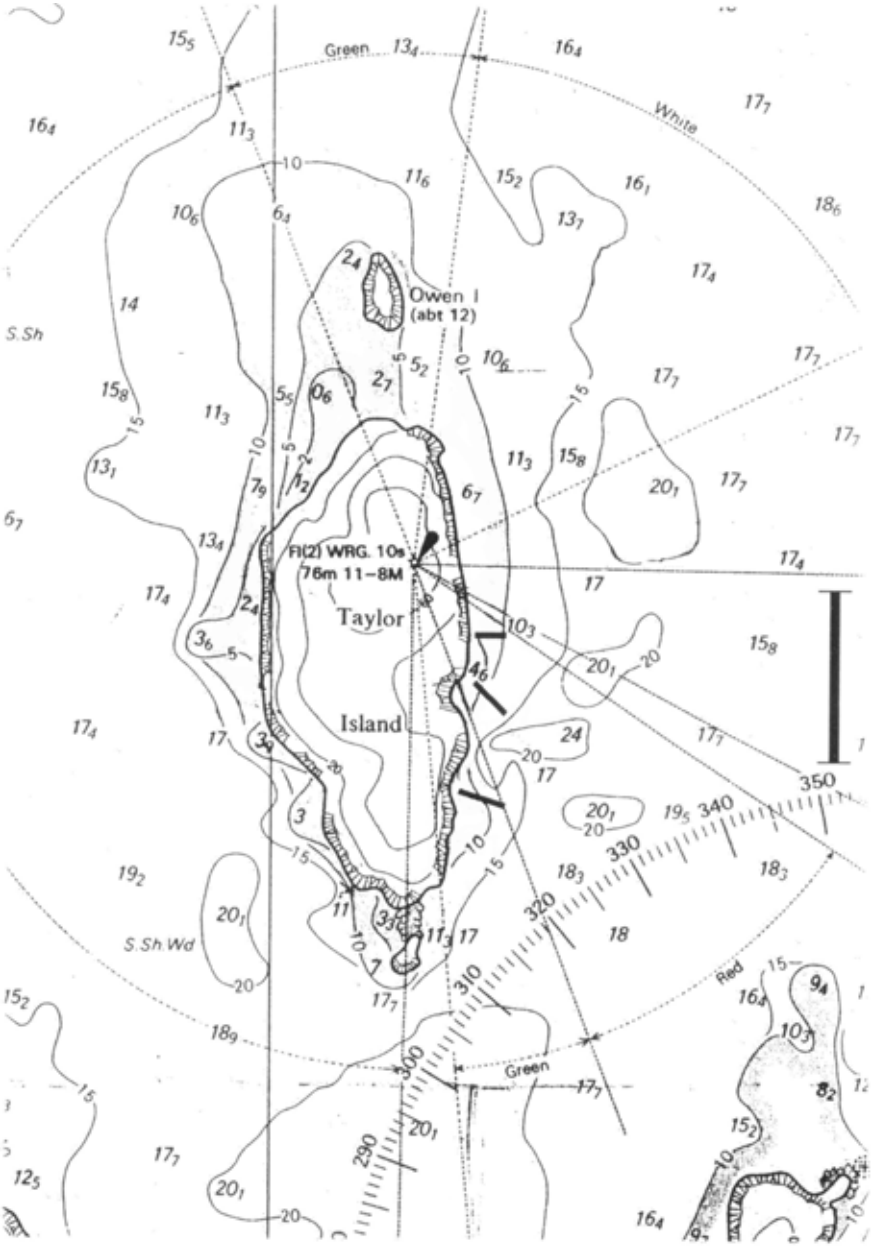

FIG. 3. Map of Taylor Island showing the three sites on the eastern side sampled in June, 2000 (bars). The edge of Grindal Island is visible in the bottom right. Scale bar = 1 km. Depths in metres.

TABLE 3. Number of *Perkinsus*-positive molluscs from Taylor and Grindal* Islands and number tested.

Mollusc	19/6/00	13/2/01	10/5/01	13/5/02*
Haliotis rubra	11/42	17/30	13/23	0/1
H. laevigata	0/8	0/5		0/3
H. scalaris	0/3	0/1		
H. roei	0/2	0/26		1/8
H. cyclobates	0/6			
Scutus antipodes (elephant snail)	0/3			0/1
Malleus sp. (hammer oyster)	0/5		0/9	
Australium aurium (turban shell)	0/9			
Hipponix (Sabia) conicus (curved limpet)	0/7	0/42		0/3
Ambichilepas nigrita (keyhole limpet)	0/1			
Granata imbricata (false abalone)	0/5			
Phasianella ventricosa (lattice snail)	0/2	0/4		
Austrocochlea odontis (checkered top)		0/10		
Austrocochlea concamerata				0/3
Austrocochlea adelaidae				0/2
Clanculus sp. (juveniles)				0/2
Turbo undulatus (common warrener)		0/26		0/17
Turbo torquatus (turban shell)		0/2		
Lepsiella sp. (whelk)	0/2	0/3		
Pleuroploca australasia				0/1
Conus anemone				0/1
Charonia lampas				0/1
Nerita atramentosa				0/9
Siphonaria diemenensis				0/7
Dicathais orbita (cart-rut shell)		0/11		0/23
Cominella lineolata		0/2		0/1
Thalatia conica		0/20		
Cellana tramoserica (variegated limpet)		0/5		0/24
Cellana solida				0/11
Patelloida alticostata				0/6
Patellid (limpet)	0/2	0/2		
Umbonium vestiarum (button top)		0/2		
Bankiva fasciata (banded bankiva)		0/1		
cockle			0/13	
scallop			0/18	
Pinna sp. (razorfish)			0/28	
Plaxiphora albida				0/3
Chiton		0/4		
	11/97	17/196	13/91	1/127

Discussion

The only mollusc found infected with *Perkinsus* at the experimental site was the blacklip abalone. Though other *Haliotis* species have been found infected in earlier work, and an

infection was found in an *H. roei* from Grindal Island, none of the other *Haliotis* species at the experimental site were infected. In terms of biomass, blacklip abalone appeared to be by far the most abundant molluscan tissue at the site, and the absence of *Perkinsus* from other molluscs sampled, strongly suggests that the *Perkinsus* parasite was being maintained solely by blacklip. Thus there was evidently no reservoir of *Perkinsus* in the immediate vicinity.

OBJECTIVE 3: TO PRODUCE AN EPIDEMIOLOGICAL MODEL

To understand the population dynamics of *Perkinsus* at the experimental site, we constructed a flow chart containing what appeared to be the main factors controlling *Perkinsus* abundance (Fig. 4).

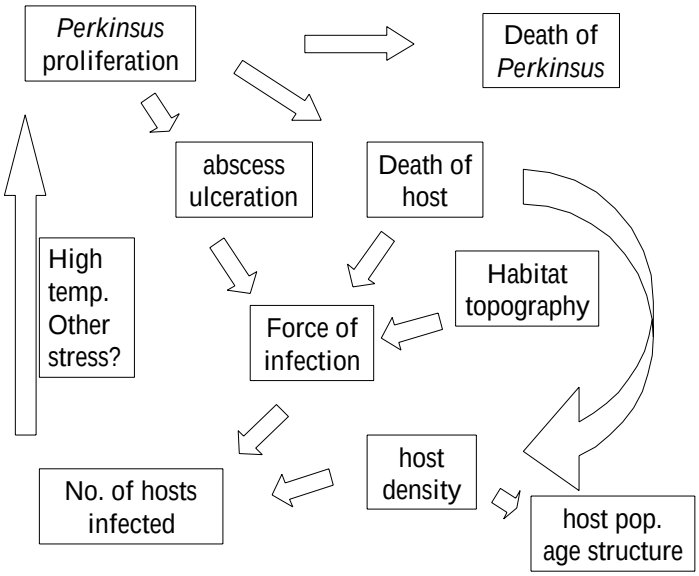

FIG. 4. Initial flow chart for the population model.

We sought to obtain data for most of the parameters. Three for which we had no information were the 'force of infection' (i.e. the likelihood that an abalone would become infected over a particular period), the density of abalone and the mortality rate of the abalone as a result of the infection.

3.1 FORCE OF INFECTION

Methods

To determine the force of infection, uninfected abalone were transferred on 3 occasions from West Bay to the test site 6 km away, and positioned in crevices to avoid predation using SCUBA or hookah. Translocations were carried out in January each year as earlier work indicated that infections were more prevalent in late summer than at other times of the year and therefore that transmission may occur during the summer.

West Bay was chosen as the source for translocated abalone because it was relatively close to the experimental site, had a good population of blacklip abalone, and commercial abalone divers considered it to be free of *Perkinsus*.

The Ray Test was used to estimate the prevalence, if any, of *Perkinsus* in West Bay abalone prior to translocation. We had planned to use frozen abalone waste obtained from processors as Goggin *et al.* (1990) had found that *Perkinsus* sp. could survive freezing and would develop in thioglycollate when the tissue was thawed thus enabling detection of the infection. We tested this phenomenon on three occasions. First, a greenlip abalone with gross signs of *Perkinsus* infection (muscle blemishes), originally collected from Taylors Landing on 3 January, 2001 (CDR 13757 3/1/2001), was thawed 10 days later and pieces incubated in thioglycollate medium for 7 days. Swollen *Perkinsus* cells developed which, though smaller than normal, were readily visualised by the Ray test. Second, a frozen blacklip with typical *Perkinsus* blemishes that had been thawed in June 2000 and refrozen, was rethawed in January 2001 and pieces cultured in thioglycollate. They were all negative using the Ray test. Unenlarged *Perkinsus* cells were found by microscopic examination but all appeared to be dead suggesting that the parasite could not withstand repeated freezing and thawing. In the third test, frozen viscera from shucked greenlip (7) and blacklip (7) from Thistle Island, some of which had had *Perkinsus* like lesions, were cultured on 15 January and examined on 18th, 19th and 24th January. No *Perkinsus* were found. Thus the results from frozen tissue appeared to be equivocal so we sought fresh material for the Ray test.

On the 19 February, 2001, pieces of digestive gland, gill, heart and haemolymph from 100 freshly collected blacklip from West Bay were cultured in thioglycollate at room temperature and examined for *Perkinsus* 4 days later. No infection was found. On the 24 January, 2003, a further 29 blacklip were taken from West Bay, tested using the Ray test and they also all proved negative. We concluded that *Perkinsus* was very rare or absent from the West Bay abalone and that any infections detected in translocated abalone at the experimental site had been acquired at the site.

Blacklip being moved from West Bay to the experimental site were tagged on board using circular coloured plastic numbered tags attached by a plastic rivet through one of the shell apertures. Of the 131 in the first trial, 13 were attached to two roof tiles in a crab pot fixed in a crevice, and the remainder were put into crevices in the vicinity. After 1 and 3 months, tagged animals were collected to determine how many had become positive for *Perkinsus* using the Ray Test. The tag numbers and abalone sizes for those recovered in 2001 are given in Appendix 5 (Tables 1 and 2).

In the second trial, 200 blacklip were tagged, moved and resampled 1 and 3 months later, and in the third trial 200 tagged blacklip were resampled after 4 months.

Results

In 2001, after 1 month at the experimental site, only 2 of 43 translocated abalone were positive for *Perkinsus* (5%; Table 4).The crab pot in which 13 tagged abalone had been placed was found broken and empty. After 3 months, 18 of 21 abalone were infected (86%). Only one tagged abalone was found after 12 months and it was infected. In 2002, 1 month and 3 months after translocation, low prevalences of infection were detected (2%

and 11% respectively). In 2003, a high prevalence was again observed in the translocated abalone (63%).

TABLE 4. Numbers of abalone translocated and numbers that subsequently became infected with *Perkinsus.*

Date tagged	Number tagged	No. infected after 1 mo.	No. infected after 3-4 mo	No. infected after 12 mo
17 Jan, 01	131	2/43	18/21 (86%)	1/1
30 Jan, 02	200	1/46	3/27(11%)	-
24 Jan, 03	200	-	30/48 (63%)	-

Infections found after 1 month were very low (less than 10 *Perkinsus* cells seen in the cultured tissue) and there were no signs of lesions. After 4 months a few abalone developed very heavy infections (hundreds of cells throughout the tissue) but still none had developed any gross signs of infection. The abalone collected after 12 months was heavily infected and had developed a 5mm diameter abscess in the muscle.

3.1.1 Loss of tagged animals

Only 1 tagged abalone was found after 12 months. While not immediately relevant to the model it was a surprising finding that needed some explanation. Three possible reasons were evaluated.

3.1.1a Death. Some tagged animals died. Ten tagged but empty shells were recovered (Table 5). This suggests that at least 5% of the translocated animals died within 4 months.

TABLE 5. Numbers of tagged empty shells recovered and total number of tags recovered during the three years.

Date tagged	Tagged dead/total tags recovered after 1 month	Tagged dead/total Tags recovered after 3-4 months
17 Jan, 01	3/46	2/23
30 Jan, 02	0/46	0/27
24 Jan, 03	-	5/53

3.1.1b. Tag lost. Some tagged animals lost their tags. Close examination of the shells of 4 abalone collected from the seabed adjacent to 41 tagged abalone, 1 month after tagging in 2001, indicated that 2 had lost their tags.

3.1.1c. Movement. Some tagged animals moved from the boulder on which they were placed.

Method:
To obtain some measure of movement we removed all accessible abalone from one quadrat, marked all abalone in another, and reexamined the quadrats after one week.

Quadrat A was a metal frame 1m^2 placed at site 2 and anchored down with rocks. Fifteen abalone were removed from this quadrat. Three small abalone were left in the quadrat because they were unable to be removed. The quadrat was examined a week later.

Quadrat B was a second metal frame 1m^2, also at site 2, and contained 17 abalone. These were marked with a lump of underwater putty and the quadrat examined after 1 week.

Results:
After 1 week, Quadrat A had acquired one large abalone. Quadrat B contained 11 marked and two unmarked abalone. Thus about 15% of abalone had apparently moved into or out of the quadrats during the week. Thus some of our tagged abalone could well have moved into concealed crevices or out of the area we searched during a year.

The putty marker was not very successful as it was difficult to recognise after a week. The two unmarked abalone may have been originally marked indicating our estimate of 15% may be an overestimate.

Discussion
In 2001 and 2003, after 3 to 4 months at the site, the majority of translocated abalone had become infected. The prevalence in the resident abalone was also high (Table 1.). In contrast, few infections were acquired in 2002 and this appears to correspond to a relatively low level of infection in the resident abalone in that year. Evidently the rate of transmission at Taylor Island is extremely high with many new infections each summer.

The poor recovery of tagged abalone after 1 year was the result of a number of factors. At least 5% of the translocated abalone died. At least 5% lost their tags each month, probably when squeezing into crevices. A double tagging experiment would give a better indication but that was beyond the scope of this project. Fifteen percent of abalone moved out of a 1 m^2 quadrat in a week suggesting many may move into concealed crevices during the year. Other losses could have occurred from fishing because the area was open to commercial abalone divers.

3.2 DENSITY OF ABALONE

As abalone were the only significant host in the area, and possibly the only host, we needed an estimate of abalone density for the epidemiological model. The experimental site had crevices in the rock and is strewn with boulders up to 2 metres across making estimates of abalone abundance extremely difficult.

On 19 June, 2000, abalone were collected from along 2 transects 1 metre wide marked by a weighted rope set at right angles to the shore and extending into the sea grass 5m deep about 20 m away., thus covering about 20 sq m each, and then abalone were collected from a third area of ideal habitat approximately 10 sq metres. The numbers collected were 1, 11 and 30 giving an overall density of 0.8 abalone per sq m.

On the 20 February, 2001, five more transects (T1-T5) using the weighted rope were run at Taylor Island (Fig. 5) and again abalone were collected in a 1 metre wide strip. T3 was situated over the tagged abalone site.
The numbers of blacklip collected in the 5 transects were 13, 1, 16, 5, and 9, i.e. 44 in 100 sq.m. = 0.4 abalone/sq.m.

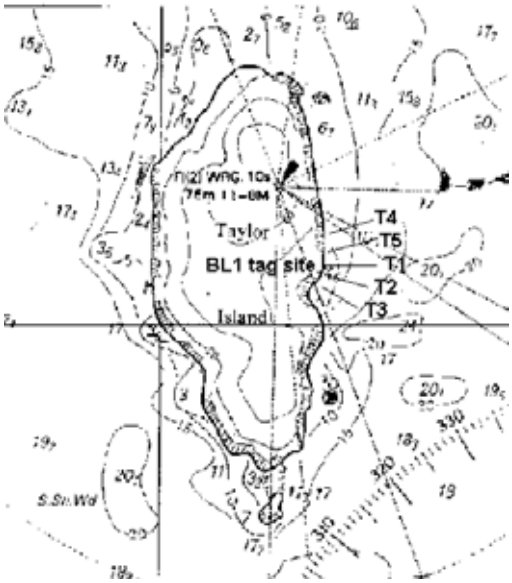

FIG. 5. Map of Taylor Island showing 5 transect sites (T1-T5) on the eastern side sampled February 20, 2001. (Co-ordinates: **T1/BL1** tag site: 34 52.702S 136 00.872E; **T2:** 34 52.733S 136 00.880E; **T3:** 34 52.755S 136 00.864E; **T4:** 34 52.644S 136 00.909E; **T5:** 34 52.673S 136 00.897E.)

On 13 May, 2002 1 m quadrats were used as they were more suitable than the weighted rope because rough weather conditions and surge meant that the transect was often a very unstable reference line. Metal quadrats were easier to handle in position under such conditions. Quadrats were replicated by randomly placing them in areas of abalone habitat at the site. In 8 quadrats were 11, all in one quadrat, giving an average density of 1.4/sq.m.

These estimates are largely based on adult blacklip. We rarely saw juveniles. Juvenile blacklip are believed to hide deep in crevices. If they carry *Perkinsus,* as they do in the laboratory, our abalone densities are underestimates.

3.3 PHYSICAL PARAMETERS OF THE EXPERIMENTAL SITE

3.3.1 Geoacoustics

The high prevalence of *Perkinsus*, the strong force of infection and the highly localised focus of infected abalone suggested that topography may be critical in maintaining the disease. With limited visibility underwater it was difficult to comprehend the topography of the site so to give a better picture, an acoustic survey was carried out on September 19, 2002.

Method

The site was mapped with a 3-dimensional bathymetric model to 1 m resolution using a Geoswath system (Geoacoustics Ltd.). Thus was a sidescan sonar fitted to the Flinders Ports catamaran "Pathfinder" . Two submarine transects were surveyed: one 4 km long by about 100 m wide in a north-south direction along the eastern length of Taylor Island, an area over basalt outcrops and sandy seagrass beds; and the second was a 200 m square transect at another known *Perkinsus* area off Hopkins and Thistle Islands, over limestone ledges and platforms. Point depth data obtained from the sonar was then supplied as ASCII files, which were converted to 3-dimensional shapefiles using ArcView Geographical Information Software, with 3D, Fly-by Animations and Spatial Analyst software extensions.

Results

Figure 6 presents a preliminary map of the Taylor Island site, showing three-dimensional topographic relief.

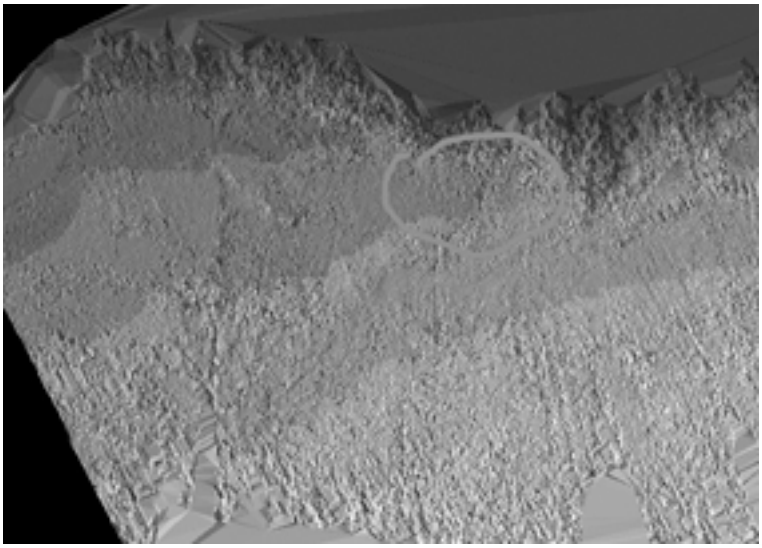

FIG. 6: A preliminary three-dimensional map of the central eastern littoral zone of Taylor Island experimental site, over a known *Perkinsus* hotspot (circled).

Discussion

The site is associated with a gully between two shallow areas. Though the resolution of the equipment is only to 1 metre, it does appear that there is sufficient change in topography to produce different hydrodynamics in the hot-spot versus adjacent areas. Eddies which bring moving algae to the abalone enabling them to thrive may also hold *Perkinsus* zoospores in the microhabitat long enough for them to reinfect abalone in the same area. Localised differences in hydrodynamics may also result in some abalone being subjected to greater extremes of temperature and/or salinity than nearby abalone and this may contribute to the high prevalence of *Perkinsus* (see below).

3.4 ABALONE MORTALITY FROM PERKINSUS

In constructing the initial flowchart (Fig. 4) we had considered that the death of infected abalone would be the main source of infective stages because in the laboratory the culture of degenerating abalone tissue led to large numbers of hypnospores and eventually zoospores. Degenerating molluscan tissue is an important component of the culture medium if it is to produce large numbers of healthy hypnospores (Earnhart et al., 2004).

The prevalence of *Perkinsus* in the translocated abalone quickly reached levels found in the resident individuals indicating a high rate of transmission, yet the resident adults were not dying from the infection, possibly because they were able to contain the infection even in the summer.

As adults were not succumbing to the infection we sought infected juveniles. Few juvenile abalone were ever seen at the site, suggesting that it was juvenile abalone killed by the parasite that were a major source of infection. We therefore measured the mortality rate of infected juvenile abalone in the laboratory.

1st experiment

Method. Juvenile, hatchery-reared abalone were obtained from a South Australian hatchery and maintained in laboratory aquaria. Groups of 7+ blacklip and 7+ greenlip were exposed to zoospores of *Perkinsus olseni* for 3 d then maintained at 15oC for up to 5 mo. Similar groups of unexposed blacklip and greenlip were maintained as controls in adjacent tanks.

Result. All 7 blacklip and 5 of 9 greenlip exposed to *Perkinsus* and that survived until the end of the experiment tested positive for *Perkinsus* showing that the disease caused little mortality at this temperature. All controls of both species of abalone tested negative for *Perkinsus*.

2nd experiment

Method. Juvenile blacklip from the NSW Brackish Water Research Station, Port Stephens, were exposed to zoospores of *Perkinsus olseni* for 3 d then maintained at 19C in the same aquaria as controls.
After 6 months the juveniles were examined for infection. After 3 months, cohabiting sub-adults were transferred to an aquarium at 24 -25C and they were examined after a further 6 months.

Result

Of the experimentally exposed juvenile abalone, 82% (28/34) tested positive for *Perkinsus* after 6 months and there was no significant mortality. None of the 55 cohabiting abalone became infected.

Conclusion

These results show that abalone and other molluscs are not necessarily killed by *Perkinsus*. They can maintain low levels of infection for months, even at temperatures of 19^{o}C and above. The finding is contrary to the view based on field observations that *Perkinsus olseni* (synonym *P. atlanticus*) is highly pathogenic (Comps and Chagot, 1987; Park and Choi, 2001) but concurs with laboratory experiments carried out by Goggin (1990) with *P. olseni* infections in bivalves.

3.5 MODEL

A simple mathematical model of *Perkinsus* infection in abalone was based on standard deterministic models of microparasite infections (Anderson & May 1979; May & Anderson 1979), modified so that release of zoospores occurs only on the death of the host, which was our understanding of the life cycle of the pathogen at the time of development of the model. Given that our experimental results showed that *Perkinsus* did not kill the abalone, its direct applicability is likely to be limited. A model developed more recently for *Rickettsia* infection of abalone, which examines the effect of fishing mortality on parasite prevalence in the presence of reserves (McCallum et al. 2005) has the potential to be adapted to deal with *Perkinsus* in Australian abalone. However, this was not possible within the timeframe of the current grant.

VARIABLES

X the number of susceptible abalone
Y the number of infected abalone
Z the number of zoospores

PARAMETERS

a recruitment per capita of abalone
b disease-independent death rate
α increment in death rate caused by *Perkinsus*
β transmission coefficient
μ death rate of zoospores
λ zoospores released per dying abalone

Assume that zoospores are released only on the death of the host.

Then:

(1.1)

(1.2)

(1.3)

μ is large in comparison with α, b etc (the life span of zoospores is very short), meaning that the zoospore population is essentially at equilibrium with current population sizes of *X* and *Y*. Hence, from (1.3),

(1.4)

Substituting this into (1.2),

(1.5)

From (1.5), infection will only be able to become established if

(1.6)

This is thus the threshold for disease establishment, and if the total host density can be kept below this level, *Perkinsus* will be eliminated from the abalone population.

In order to apply such a model in practice the components of Eqn (1.6) must be estimated. The difficult parameter to estimate is the transmission rate β. This is a problem common to most epidemiological models. However, what can be estimated is the component β*Z* of Eqn (1.1). This is Λ, the force of infection (the rate at which uninfected abalone pick up infection per unit of time). We conducted experiments to estimate this in the field. As is shown below, we can use these estimates to derive the threshold abalone density at which *Perkinsus* infection will be eliminated, provided we also estimate of *X* and *Y* (estimating *Z* in the field is likely to be very difficult, though see Discussion below).

From Eqn. (1.4),

(1.7)

And from Eqn. (1.6),

, (1.8)

where N_T is the threshold density. Thus,

(1.9)

As the death rate α of abalone due to infection was very low, and abalone are long-lived in the absence of infection (i.e. *b* is small), whereas our estimates of Λ are much higher, Eqn (1.9) suggests that the threshold host density will be much lower than the observed density of infected abalone *Y*. Using this model, elimination of the infection by reducing abalone density was therefore unlikely to be a feasible management strategy.

Our experimental results suggest that the principle source of infection is not from abalone dying from the infection but from infected abscesses that rupture and release zoospores.

Abalone with abscesses are highly localised in the environment. A useful area for future research would be to develop a spatially-explicity model that could be used to evaluate the potential of removal of abalone from these localised areas as a central strategy.

3.6 GENERAL DISCUSSION

To generate the model of the population dynamics of *Perkinsus* in the area, we used the flow chart incorporating what appeared to be the main factors influencing the spread of infection (Fig. 4). The parameters we measured included: force of infection (3 of 87 infected after 1 month, 51 out of 96 infected after 3.5 months); prevalence in resident animals at site (36%); density of abalone (0.6 abalone/sq m.); prevalence of abscesses in infected resident abalone (39%); and time taken for abscess to develop (approximately 1 year). A surprising result from our measurements was that the death of host as a result of infection appeared to be close to zero.

We had hypothesised that the main source of infection was from zoospores released from dying and dead abalone. However, as we found that *Perkinsus* did not kill the abalone, the mathematical model we constructed was not immediately applicable. Additional parameters needed to be measured and incorporated before we could understand the dynamics of the parasite populations. The time frame of the project precluded this.

It is possible that ruptured infected abscesses form a source of infection. Abscesses are common in infected older abalone and sometimes they contain hypnospores (Goggin and Lester, 1995). In cultured abalone tissue we have observed that as hypnospores swell, they are forced out of the tissue onto the external surface. Thus it seems likely that in the wild when an abscess ruptures, hypnospores are released, come into contact with seawater and are able to continue their development. This seems an excellent highly-evolved mechanism for producing infective zoospores without killing the abalone. It is noteworthy that nodules containing *Perkinsus* cells are found on the gills of infected clams in Portugal (Azevedo, 1989) and Japan (Choi et al., 2002). If hypnospores can develop within these nodules also, then this provides a common mechanism of transmission for *P. olseni*.

It may not be the whole story however, because blacklip abalone with *Perkinsus* (presumably *P. olseni*) in New South Wales do not apparently exhibit abscesses, nodules or pustules (D. Worthington, NSW Fisheries, pers. com.) suggesting transmission there is not by this route. Perhaps mortalities from other causes in NSW enable transmission from infected abalone.

A third hypothesis is that infected abalone are eaten by fish. The parasite swells in the gut of the fish and eventually forms zoospores when the hypnospores are released into seawater with the faeces. This capability has been demonstrated for *P. marinus* in North America (Hoese, 1964) but has not yet been investigated in *P. olseni*.

Thus, measurements on zoospore production from abscesses, and evaluation of spore production after predation by fish need to be undertaken to refine the model to make it usefully predictive.

The results of this project lead us to consider a shift in the paradigm that *Perkinsus* is a primary pathogen: it instead appears to be a ubiquitous opportunist that is either

eliminated by or sequestered in healthy abalone, but proliferates in individuals stressed either by climatic conditions approaching the species' thermal tolerance limits, or other factors such as starvation, sudden rises or falls in temperature or salinity. Handling stress such as exposure to bright light, vibrations, shaking or dehydration may also affect the animals' resistance. In our first translocation, 18 of 21 animals became infected (86%) compared to only 13/23 (57%) in the resident abalone, a significant difference at the 95% level of confidence, indicating that the translocated animals were more susceptible than the resident animals, possibly because of the stress of the move.

Sudden increases or decreases of sea surface temperature may predispose the abalone to infection. The maximum sea surface temperature at the experimental site dropped 3^{o}C in the period from 2001 to 2002 [source: CSIRO Marine Research Hobart, satellite data]. This reflects, and may have been closely linked to the cause of, the sharp decline in prevalence of *Perkinsus* (from 85% to 11%, respectively) that we observed between 2001 and 2002. There may also be irregular deepwater upwellings or cold, internal waves that plunge particular rocky reefs and semi-enclosed bays under very cold water several times with each outgoing tide (A.J. Pile, University of Sydney, personal communication).

Physical changes in the environment affect algal production. Blacklip abalone feed on drifting algae thus a reduction in algal production may stress the animals by reducing their accessibility to food. This would be exacerbated in certain areas because of the way local topography affects eddies.

There are anecdotal reports of changes in the bottom flora of the Gulf of St Vincent prior to the mass die-off of greenlip abalone in the mid 1980s. The epizootic of *Perkinsus* in Greenlip Abalone at this time (Lester, 1994) may have been an indicator of abalone stress and starvation rather than the primary cause of death.

This hypothesis is supported by recent publications that detail a mechanism by which stresses (mechanical agitation, sudden heat and cold) reduce molluscan immunity. The primary line of molluscan defense - phagocytosis of opportunistic pathogens by hemocytes - is impaired after the release of stress hormones, specifically catecholamines such as noradrenaline, in a dose-dependent manner. Malham *et al*. (2003) have now shown this in the European abalone, *Haliotis tuberculata*.

Methods are now available to test for *Perkinsus* zoospores in the water column. As we found the 'force if infection' to be very high, then we should be able to detect and quantify the abundance of *Perkinsus* cells in the water column using a technique such as real-time PCR. This may demonstrate whether the recognised foci of infection occur where the force of infection is greatest, or whether the infective stages are widespread (which we suspect) and only compromised abalone exhibit overt signs of disease. Quantification of zoospores in the water column will also provide an estimate of *Z*, the number of zoospores, in the mathematical model.

Our main conclusions from the project then are that *P. olseni* is frequently a symbiont of abalone rather than a primary pathogen, and that infections probably indicate that the abalone have been immunocompromised by some other factor. Possibly very heavy *Perkinsus* infections, heavier than we could induce in the laboratory, could cause death, if only because of impaired haemolymph circulation from the aggregations of *Perkinsus*

cells, but we believe such heavy infections only arise when the animals are stressed by some other factor.

OBJECTIVE 4. TO RECOMMEND APPROPRIATE MANAGEMENT METHODS

The parasite does not normally kill the abalone. The abscesses in older animals appear to be chronic sources of infection for younger animals. The current hypothesis thus suggests that removal of the older animals may reduce the prevalence. However, removal of older animals has been carried out by abalone divers for decades without apparently influencing the prevalence of the parasite (though it has not been measured). It would be valuable to make a concerted effort to remove all very large abalone from the experimental site (perhaps before the quota season opens in November) and then to measure the new force of infection, possibly using real-time PCR. A reduction in zoospore abundance may be immediately obvious from PCR results. A reduction in infections in translocated abalone may be evident after 3 months and prevalence in resident animals after 2 years.

5.0 ANCILLARY RESEARCH

5.1 NSW surveys for Perkinsus

In a subsidiary project, two surveys were conducted in NSW to determine what reservoir hosts were present at the apparent 'front' of the disease where abalone mortalities and *Perkinsus* prevalences were high. This was in and around Jervis Bay.

The surveys found *Perkinsus* only in blacklip abalone at the edge of the die back where abalone had already become difficult to find (Table 5).

The absence of abscesses from NSW abalone may be related to the rapid spread of the infection and the high mortality of abalone in the infected areas. The initial infection may have come from a reservoir host such as the blood cockle, *Anadara trapezia*. Though cockles were not found infected in our NSW survey, they are frequently infected with *Perkinsus olseni* in Moreton Bay, Queensland (Goggin, 1990; Murrell *et al*., 2002).

TABLE 5. Prevalence of *Perkinsus* in molluscs from the NSW coast. For details see Gill (2003).

Locality	Date	Species	Sample size	No. infected
Jervis Bay	12 Nov 2001	*Haliotis rubra*	32	12
		Turbo torquatus	12	0
		Australium tentiforme	5	0
	24-25 June,'03	*H. rubra*	114	2
		H. coccoradiata	1	0
		Anadara trapezia	152	0
	10 July, 2003	*H. rubra*	4	1
		T. torquatus	3	0
		renellid	2	0
		Australium sp.	1	0
		?Monilea sp.	1	0
Port Stephens	1-7 April,'03	*H. rubra*	40	0
		H. coccoradiata	23	0
		Pinna bicolor	39	0
		Scaeochlamys sp.	21	0
		A. trapezia	47	0
		Trichomya sp.	40	0
		Donnax deltoides	40	0
Total			577	15

5.2 Development of non-infectious positive material for the Ray test

Dr Judith Handlinger, Tasmanian Department of Agriculture, and Dr Duncan Worthington, NSW Fisheries, requested positive control material for the Ray test that was not infectious. We therefore carried out a series of tests to evaluate options for non-contagious control material.

Perkinsus trophozoites made non-viable with formalin, irradiation or colchicine all failed to swell in thioglycollate. They remained negative by the Ray test and thus were no use as a positive control. Live cells that had swollen in thioglycollate and then killed by freezing, ethanol or formalin retained their iodinophilic properties and could therefore be used as a partial positive control. The results have been published (Moore *et al.,* 2002; Appendix 6).

5.3 Sensitivity of Perkinsus to UV light

We were requested by a commercial company to test the sensitivity of *Perkinsus* to ultraviolet irradiation. We found that UV radiation in excess of 60,000μWs/cm^2 inhibited most hypnospores and probably rendered all zoospores non-viable (Moore and Lester,

Appendix 7). This level is in excess of what is required for most bacteria but the same order of magnitude as that required for other protozoa.

BENEFITS

The information obtained during the project benefits primarily the fisheries managed by South Australia (90%) and NSW (10%) as indicated in the original application. We have shown that abalone mortalities in South Australia are unlikely to be caused by *P. olseni* and other causes must be sought.

The abscesses formed around *Perkinsus* cells plague processors for two reasons. They are aesthetically undesirable, and they represent infection by an organism listed as a dangerous pathogen by the international OIE (Office International des Epizooties). Our work suggests solutions to these two aspects. First that old heavily infected animals be removed from hotspots to decrease the 'force of infection'.

Secondly, our molecular work demonstrated that *Perkinsus olseni* and *P. atlanticus* are synonyms and therefore the parasite is widely distributed across the old world (Australasia, Korea, Japan, Portugal, Spain). The low pathogenicity and wide distribution lead to the recommendation that *P. olseni* be removed from the OIE list of dangerous pathogens, thus freeing up the export of live and chilled Australian abalone. Acceptance that the parasite is non-pathogenic would also free up movement within Australia to the benefit of Tasmanian processors because chilled product from South Australia was prevented from being shipped to Tasmania for processing because of the risk of *Perkinsus*.

Results of the project as it progressed were communicated to the industry and to other groups informally, and also in formal presentations four to the industry and five at scientific meetings.

Presentations to the abalone industry:

Lester, Kleeman, Barker and McCallum 2001 Epidemiology of *Perkinsus olseni*, pathogen of abalone. Presentation to the National Abalone Convention, Adelaide, 19-21 August, 2001. Later issued by the Abalone Association on a CD.

Hayward and Lester. 2003. Blacklip abalone and Perkinsus: An Update. Special meeting of NSW Fisheries, Cronulla, March 2003.

Hayward 2003. Blacklip abalone and *Perkinsus*: what's ahead? Special meeting of NSW Fisheries, Cronulla, September 2003.

Hayward and Lester 2004. Epidemiology of perkinsosis in abalone in South Australia and in the lab. FRDC Abalone Health Workshop, Corus Hotel, Sydney, Sept 30-Oct 1.

Other presentations:

Lester, Kleeman, Barker and McCallum 2001 Epidemiology of *Perkinsus olseni*, pathogen of abalone. Bienniel meeting of the European Association of Fish Pathologists, Dublin 9-14 September, 2001 (Oral).

Hayward CJ, Lester RJG, Barker S, McCallum H, Murrell A, Kleeman S. *Perkinsus olseni*: transmission among wild blacklip abalone in South Australia5th

Symposium on Diseases in Asian Aquaculture, Nov 25-29 2002, Gold Coast, Australia (Oral).

Hayward CJ, Lester RJG. 2003 Current status of perkinsosis among blacklip abalone in Australia, with a fresh look at transmission of the disease. 1st FRDC Aquatic Animal Health Subprogram Scientific Conference, 8-10 Oct 2003 Geelong (Oral).

Hayward CJ, Lester RJG. 2003 Current status of perkinsosis among blacklip abalone in Australia, with a fresh look at transmission of the disease. 5th International Abalone Symposium, Oct 2003, Quindao, China (Oral).

Hayward CJ. 2004. Perkinsosis: A New Take. School of Aquaculture departmental seminar, September 15 2004, University of Tasmania, Australia.

FURTHER DEVELOPMENT

Abalone may develop a heavy infection with *P. olseni* only when they are stressed. It would be valuable to monitor the environment at known disease foci to determine whether, for example, cold subsurface waves move into the foci and thus stress the abalone, or whether infection is associated with food limitation. The parasite is not normally pathogenic to the abalone but its presence appears to be a good indicator that a chronically infected animal has been under some form of stress.

Our results suggest that removal of older animals from a disease hotspot will decrease the force of infection and ultimately the prevalence in resident animals. It would be useful to now test this hypothesis in the field.

It is still not clear how the parasite is transmitted. Almost certainly broken abscesses are an important part. It is possible that fish eat infected abalone, the parasite develops in the gut, and eventually forms infective zoospores when released to sea water in the faeces of the fish. These hypotheses need to be tested in the laboratory and the data incorporated into a revised mathematical model of the *Perkinsus* population..

PLANNED OUTCOMES

The planned outcome was a mathematical model of the *Perkinsus* population at the experimental site. The model provided a conceptual framework incorporating the main parameters which were thought to affect *Perkinsus* abundance. Ideally, the model would then be used to predict how changes in parameters would affect the prevalence of the parasite.

Measurement of the parameters showed that a basic assumption of the model was incorrect. We had assumed, in agreement with much of the literature on *Perkinsus*, that the parasite killed the host. Our measurements indicated that at least with blacklip and greenlip abalone, mortality as a result of infection with *P. olseni* was negligible. Alternative parameters influencing the force of infection thus need to be examined. As hypothesised in the flow chart (Fig. 4), abscess ulceration, a consequence of the abalone immune defence mechanism, may be far more important than first appears. Some measure of this is now needed for the model to be progressed.

Though the details of the model are mathematically incomplete, it is now evident that there is no significant reservoir host other than blacklip abalone at the experimental site and therefore remedial action built around blacklip is likely to be effective.

CONCLUSION

There were four original objectives.

1. To determine the extent and nature of *Perkinsus* infection around Taylor Island.

This objective was carried out by the transects done in the winter and the summer. It showed that the prevalence remained high (around 50%) for most of the year, though it was lower in the 2nd year compared to the first and third years.

2. To identify possible reservoir hosts.

767 individuals representing 38 species were examined. All but one of the 135 infections detected were in blacklip abalone *Haliotis rubra*. The exception was an infection in *H. roei*. Thus we conclude that there was no significant host in the experimental site other than blacklip.

3. To produce an ecological model

To produce the model we measured the force of infection three times. We found that within 3-4 months 50% of introduced abalone had picked up the infection. This was slightly higher than the level in the resident animals (36%). We conclude that the system is highly dynamic with high chances of reinfection each year.

In the laboratory we examined the mortality rate of experimentally infected juveniles and found that there was no significant mortality within 6 months. We conclude that host mortality as a result of the infection is not an important component in the epidemiology of *Perkinsus* at the experimental site. Other parameters such as abscess rupture need to be considered. In relation to this, we observed that abscesses did not appear for at least 4 months and may take a year to form.

4. To recommend appropriate management methods.

We recommend that the largest blacklip abalone be removed from the hotspot and then the force of infection to be remeasured. This should be evident within 3 months in introduced abalone though it may take two or more years for the prevalence to decrease in the resident animals.

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APPENDIX 1

Intellectual property

The intellectual property in the report belongs to the authors and FRDC except where indicated by reference to the work of others.

APPENDIX 2.

Staff engaged on the project

Abalone Divers Association: Mr Michael Tokley, (Executive Officer,)
Mr Bill Ford, Mr Philip Penalurick, Mr Barry Hockaday, Mr Jeff Garnaut

SARDI Port Lincoln Marine Science Centre: Mr Peter Preece, Mr Brian Foureur, Mr Thor Saunders, Dr Kate Rodda

PIRSA/SARDI in Adelaide: Dr Richard McGarvey

University of Queensland: Prof Bob Lester, Dr Sarah Kleeman, Dr Craig Hayward, Dr Anna Murrell, Assoc.Prof. Steve Barker, Assoc. Prof. Hamish McCallum, Mr Eric Boel, Mr Brad Moore, Ms Belinda Gill.

APPENDIX 3

Murrell, A., S.N. Kleeman, S.C. Barker and R.J.G. Lester. 2002 Synonymy of *Perkinsus olseni* Lester & Davis 1981 and *Perkinsus atlanticus* Azevedo, 1989 and an update on the phylogenetic position of *Perkinsus*. Bulletin of the European Association of Fish Pathologists 22: 258-265.

This appendix is not included. Please see the original publication in the Bulletin of the European Association of Fish Pathologists and the correctly printed Fig. 1 in the same Bulletin vol. 22 no.6 p. 408.

APPENDIX 4

Prevalence of *Perkinsus* in blacklip from Taylor Island, May 2001

Date	Length (mm)	Postules	Gills	DG	Muscle	Perkinsus
10/05/2001	**170**	N	0	0	0	
	160	Y	3	0	2	POSITIVE
	154	Y	2	0	1	POSITIVE
	150	N	0	0	0	
	148	N	0	0	0	
	147	Y	2	2	2	POSITIVE
	147	N	1	0	0	POSITIVE
	143	N	3	3	3	POSITIVE
	140	N	0	0	0	
	138	N	1	0	0	POSITIVE
	134	N	2	0	0	POSITIVE
	129	Y	1	0	5	POSITIVE
	127	N	2	0	0	POSITIVE
	113	N	0	0	0	
	109	N	4	2	3	POSITIVE
	99	N	0	0	0	
	98	Y	4	0	5	POSITIVE
	92	N	4	0	0	POSITIVE
	81	N	0	0	0	
	77	N	1	0	1	POSITIVE
	66	N	0	0	0	
	55	N	0	0	0	
	45	N	0	0	0	
Total	23	5	13	3	8	13

Blacklip collected from Taylor Island on the 10th of May, 2001, and tested for the presence of Perkinsus in FTM. N = no pustules. Y = pustules present. Abalone marked in bold were from quadrat 1.

APPENDIX 5

Occurrence of *Perkinsus* in translocated abalone, 2001.

Table 1. Tagged blacklip exposed to *Perkinsus* at Taylor Is. over a 4 week period Jan-Feb., 2001.

Date	Tag #	Gill	DG	Muscle	Sediment	Length (mm)
13/02/2001	500	N	N	N	N	130
	502	N	N	N	N	135
	504	N	N	N	N	105
	507	N	N	N	N	130
	508	N	N	N	N	125
	509	N	N	N	N	130
	527	N	N	N	N	140
	528	N	N	N	N	105
	529	N	N	N	N	130
	532	**POS**	N	N	N	125
	536	N	N	N	N	125
	547	N	N	N	N	120
	549	N	N	N	N	125
	553	N	N	N	N	125
	558	N	N	N	N	135
	565	N	N	N	N	120
	570	N	N	N	N	130
	572	**POS**	N	N	N	160
	577	N	N	N	N	130
	581	N	N	N	N	130
	588	N	N	N	N	105
	594	N	N	N	N	130
	602	N	N	N	N	135
	606	N	N	N	N	120
	608	N	N	N	N	115
	609	N	N	N	N	125
	611	N	N	N	N	130
	618	N	N	N	N	135
	619	N	N	N	N	125
	623	N	N	N	N	85
	639	N	N	N	N	140
	644	N	N	N	N	
	645	N	N	N	N	115
	650	N	N	N	N	130
	652	N	N	N	N	130
	662	N	N	N	N	120
	670	N	N	N	N	135
	673	N	N	N	N	130
	689	N	N	N	N	135
	694	N	N	N	N	115
	698	N	N	N	N	140
	NT	N	N	N	N	120
	NT	N	N	N	N	122
	540	empty shell				130
	544	empty shell				140
	591	empty shell				125
20/02/2001	550	N	N	N	N	150
	610	N	N	N	N	65

POS = positive for *Perkinsus*. N = negative. NT = no tag. DG = digestive gland. Length = shell length.

APPENDIX 5 (continued)

Table 2. Tagged blacklip exposed to *Perkinsus* at Taylor Island over a 4 month period.

Date	Tag #	Gill	DG	Muscle	Sediment	Length (mm)
10/05/2001	568	3	N	N	N	140
	686	2	1	1	N	150
	597	5	N	1	2	130
	665	3	N	N	N	135
	576	2	N	N	N	135
	524	1	N	N	N	120
	580	5	1	1	3	135
	666	N	N	N	N	120
	520	4	3	N	N	115
	539	1	1	N	N	120
	640	3	N	2	1	95
	693	2	N	N	N	100
	562	1	N	N	N	90
	513	N	N	N	N	85
	519	3	1	N	N	100
	595	2	N	1	N	130
	684	1	N	N	1	100
	535	N	N	N	N	100
	671	1	N	N	N	85
	585	3	N	N	N	80
	589	2	N	N	N	75
	617		EMPTY SHELL			130
	628		EMPTY SHELL			160
No. hosts infected:		18/21	5/21	5/21	4/21	

Infection in each tissue type is ranked on a scale of 1 to 5:
1 = 1 – 10 cells;
2 = 10-100 cells total in clusters fewer than 5;
3 = >100 cells in clusters numbering 1-10;
4 = >100 cells in clusters numbering 10-50;
5 = cells throughout tissue.
N = negative. DG = digestive gland.

APPENDIX 6

Moore, B.R., S.N. Kleeman and R.J.G. Lester. 2002. The development of a positive non-infectious control for the detection of *Perkinsus* using the Ray test. Journal of Shellfisheries Research 21: 871-873.

The paper is not included. Please see the original article in the Journal of Shellfisheries Research.

APPENDIX 7

The effect of UV irradiation on *Perkinsus olseni*

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Introduction

Abalone farms near to areas where wild abalone are infected with *Perkinsus olseni* risk taking the parasite in the water intake. To avoid this, one farm uses UV treatment of the incoming water. As there were no data on the susceptibility of *Perkinsus olseni* to UV, we examined the parasite for its resistance to UV to determine what dosages were required to inactivate it.

Methods

Hypnospores were tested for viability. Zoospores were not used because of the difficulty of locating them after exposure. Pieces of mantle, foot, digestive gland and gill from Anadara trapezia, from Wynnum, SE Queensland, were incubated in 20ml fluid thioglycollate medium at room temperature (approximately 24°C) for c. 7 days to facilitate hypnospore development. The medium was supplemented with 200mg chloromycetin and 200 units of mycostatin (Ray 1966). The resulting hypnospores were then excised from the surrounding tissue and placed in Petri dishes containing seawater for UV exposure. The UV source used was a UVTA Model LC 20 (Utraviolet Technology of Australasia, Adelaide), a flowthrough system designed for drinking water. Its lamp (20Watt, P/N 001-0302022 1256-L40) emitted UV radiation around 253nm and the water in the adjacent tube received approximately 30,000 microwatts/sec/cm^2 (UVTA, pers. com.). The centre of the tube was 21 mm from the glass of the lamp.

Hypnospore sensitivity was tested in three ways. In the first, the vertical inlet tube of the LC 20 unit was blocked with an opaque plastic plug near the centre of the lamp. The lamp was turned on and allowed to warm up for at least 1 min. Then 10ml seawater containing hypnospores was poured into the inlet tube using a glass funnel. After a fixed number of seconds the tube was turned off and the hypnospores poured into a petri dish. Exposed hypnospores were incubated in the light at room temperature (24°C), the seawater being replaced twice daily. Viability was assessed using a single criterion: the occurrence of cell division. After each test, the apparatus was thoroughly rinsed with deionised water to explode any adhering Perkinsus cells.

In the second method, the inlet tube was blocked near the base of the apparatus about 10mm behind the lamp filament. The lamp was turned on and allowed to warm up for at least 1 min to achieve maximum UV output. The inlet tube was then filled close to

the top with seawater containing hypnospores, sealed, and continually inverted in 2 second cycles so that the trapped air stirred the contents to simulate movement through the tube until the desired dosage was achieved. After exposure, the seawater was poured into a glass beaker, allowed to settle, and hypnospores transferred to a glass Petri dish to incubate as before. Viability was assessed using two criteria: the occurrence of the first cell division, and the presence of motile zoospores within the hypnospores. Both could be determined using a dissecting microscope without moving the hypnospores from the dishes. After each test, the apparatus was rinsed with deionised water.

In the third method, the LC 20 was laid horizontally and a Petri dish, 52 x 12mm, containing hypnospores in 6mm seawater was placed directly under the lamp so that the parasites were 15mm from the glass of the lamp, and 130mm from the lamp filament measured along the lamp, i.e. one third of the distance along the lamp. The lamp was turned on and allowed to warm up for at least 1 min to achieve maximum UV output. During the lamp warm-up, the dish was shielded by a piece of aluminium foil which was removed at the start of the exposure time. Exposure ended when the lamp was switched off. Viability was assessed using the two criteria described in Method 2.

Results

Method 1.

From these results it is evident that UV exposure of approximately 960,000 $\mu Ws/cm^2$ prevented the proliferation of approximately 95% of hypnospores exposed (Table 1. Fig. 1). Exposure at 60,000 – 120,000 $\mu Ws/cm^2$ seemingly had no effect on hypnospore viability, providing similar numbers of viable hypnospores to those of the controls.

Method 2.

Irradiation by UV had a significant effect on hypnospore viability at dosages over 60,000 $\mu Ws/cm^2$ (Table 2. Fig. 2). Hypnospores were completely inactivated by UV irradiation at dosages 480,000 $\mu Ws/cm^2$ or greater. One viable hypnospore was witnessed in one 240,000 $\mu Ws/cm^2$ exposure. Motile zoospores were seen in all hypnospores that underwent cell division.

Method 3.

Irradiation by UV had a significant effect on hypnospore viability at dosages over 100,000 $\mu Ws/cm^2$ (Table 3. Fig. 3). Hypnospores were completely inactivated by UV irradiation at dosages of 400,000 $\mu Ws/cm^2$ and greater. All hypnospores that underwent cell division formed motile zoospores.

Discussion

The UV exposure varies not only according to the time but also in relation to their position near the lamp and any shadows cast by particles in the water or edges, plugs or imperfections in the container. In Method 1, it is likely that many hypnospores were sheltered from the UV light by shadows and refraction. Nevertheless it is clear that hypnospores can survive low doses of UV but are rendered inactive by high doses.

Method 2 was probably closest to the situation when the equipment would have been functioning in a commercial setting. After 2 seconds (60,000 $\mu Ws/cm^2$) less than 10% on

average of the hypnospores were viable. Thus if the flowthrough speed was 2 or more seconds then most hypnospores would be killed.

In a commercial setting it is unlikely that many hypnospores would be carried into the inlet pipe. Hypnospores are denser than seawater and tend to drop to the bottom. They are also slightly sticky and tend to adhere to substrates. They eventually release zoospores and it is the zoospores which appear to be the main dispersal phase for the parasite. We were unable to test zoospores because of the difficulty of producing zoospores of a specific age, and then of finding them in the test solutions. It is likely that they are much more sensitive to UV than the hypnospores because their cell wall is many times thinner than that of a hypnospore.

The main phase of *P. olseni* found within abalone, the trophozoite, also has a cell wall much thinner than a hypnospore. Bushek and Howell (2000) tested the UV sensitivity of trophozoites of *P. marinus* in culture media and in filtered seawater and found that dosages as low as 5,000μWs/cm^2 affected viability and almost totally inhibited their multiplication.

We showed that few of the relatively resistant hypnospore stage were viable after exposure to 60,000μWs/cm^2. Thus it is likely that commercial equipment that gives a dose of 60,000μWs/cm^2 would almost totally prevent the passage of viable *P. olseni* cells.

Acknowledgements

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Ray, S.M. 1966 A review of the culture method for detecting *Dermocystidium marinum*, with suggested modifications and precautions. Proceedings of the National Shellfisheries Association 54: 55-69.

Table 1. Effects of ultraviolet light exposure on the viability of *Perkinsus olseni* hypnospores, placed into the inlet tube of the LC 20 UV unit with seawater.

Duration of dose (secs)	Estimated dose ($\mu Ws/cm^2$)	Non-viable	Viable	% Viable
Control	0	9	63	87.5
	0	4	78	95.1
	0	6	68	91.9
0	0	6	59	90.8
	0	3	80	96.4
	0	7	65	90.3
2	60,000	12	62	83.8
	60,000	6	76	92.7
	60,000	5	65	92.9
4	120,000	18	71	79.8
	120,000	14	70	83.3
	120,000	9	56	86.2
8	240,000	35	36	50.7
	240,000	24	34	58.6
	240,000	30	42	58.3
16	480,000	94	7	6.9
	480,000	48	4	7.7
	480,000	57	4	6.6
32	960,000	68	1	1.4
	960,000	60	0	0
	960,000	46	3	6.1

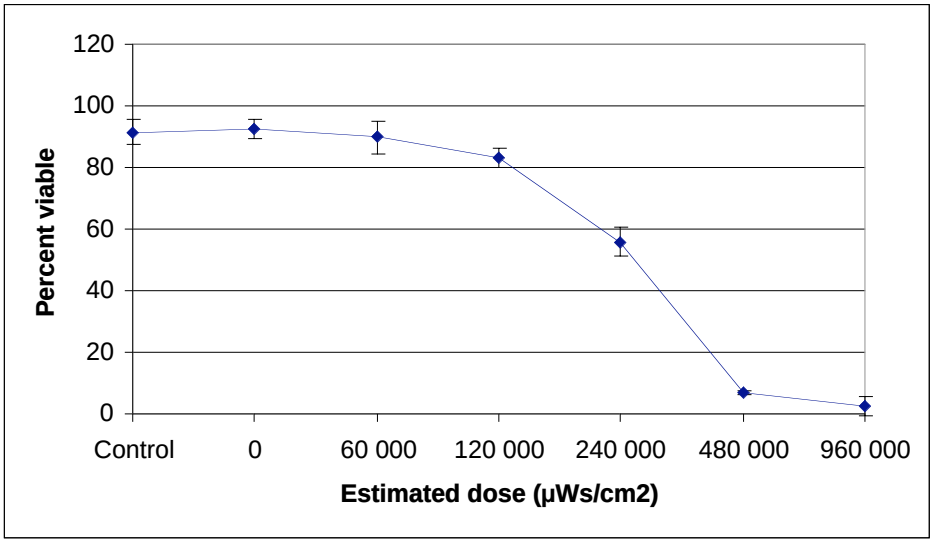

Fig. 1 Effects of ultraviolet light exposure on the viability of *Perkinsus olseni* hypnospores, placed into the inlet tube of the LC 20 UV unit with seawater. Bars represent standard deviations.

Table 2. Effects of ultraviolet light exposure on the viability of *Perkinsus olseni* hypnospores, placed into the inlet tube of the LC 20 UV unit with seawater. The inlet tube was periodically inverted in 2 second cycles.

Duration of dose (secs)	Estimated dose (µWs/cm2)	No. hypnospores that underwent cell division	No. hypnospores containing motile zoospores	Non-viable	% Viable
0	0	48	48	11	81.36
	0	51	51	6	89.47
	0	61	61	9	87.14
2	60,000	2	2	36	5.26
	60,000	4	4	43	8.51
	60,000	6	6	45	11.76
4	120,000	0	0	53	0
	120,000	1	1	41	2.38
	120,000	4	4	47	7.84
8	240,000	1	1	48	2.04
	240,000	0	0	46	0
	240,000	0	0	38	0
16	480,000	0	0	39	0
	480,000	0	0	34	0
	480,000	0	0	47	0
32	960,000	0	0	46	0
	960,000	0	0	42	0
	960,000	0	0	63	0

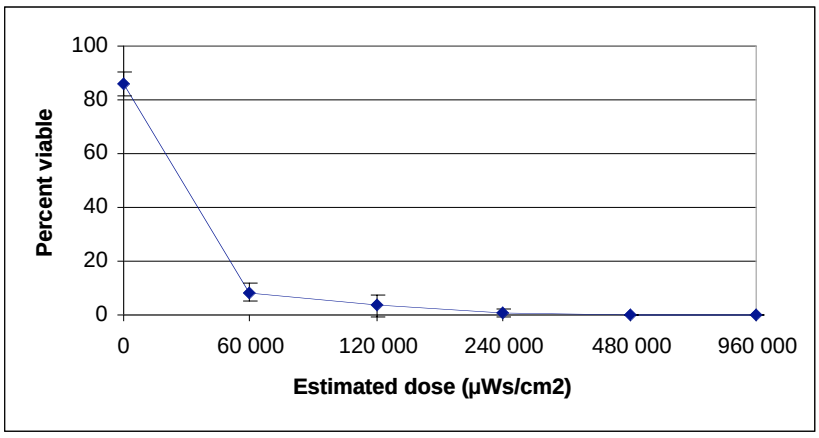

Fig. 2. Effects of cumulative dose of ultraviolet light on the viability of hypnospore stages of *Perkinsus olseni*, placed into the periodically inverted inlet tube with seawater. Bars represent standard deviations.

Table 3. Effects of ultraviolet light exposure on the viability of *Perkinsus olseni* hypnospores, under direct exposure in Petri dishes.

Duration of dose (secs)	Estimated dose (µWs/cm2)	No. hypnospores that underwent cell division	No. hypnospores containing motile zoospores	Non-viable	% Viable
0	0	67	67	5	93
	0	83	83	17	79.5
	0	56	56	7	89
2	100,000	4	4	52	7.1
	100,000	41	41	134	23
	100,000	9	9	52	15
4	200,000	7	7	56	11
	200,000	2	2	81	2.4
	200,000	3	3	42	6.7
8	400,000	0	0	76	0
	400,000	0	0	67	0
	400,000	0	0	43	0
16	800,000	0	0	78	0
	800,000	0	0	76	0
	800,000	0	0	48	0
32	1,600,000	0	0	56	0
	1,600,000	0	0	68	0
	1,600,000	0	0	39	0

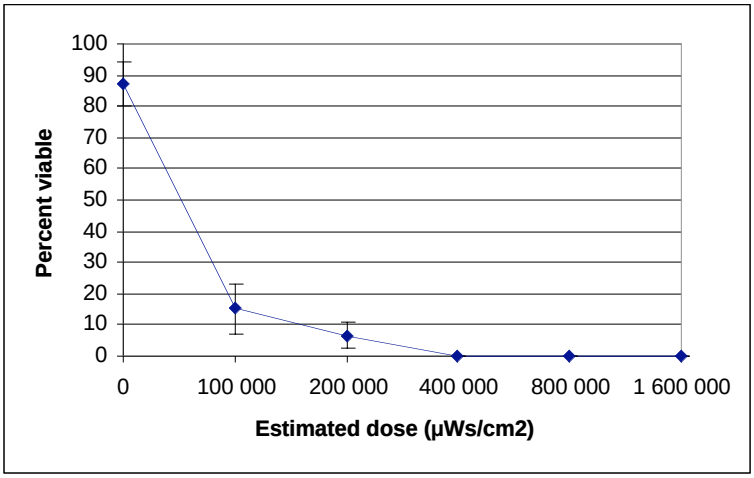

Fig. 3. Effects of cumulative dose of ultraviolet light on the viability of hypnospore stages of *Perkinsus olseni*, under direct exposure in Petri dishes. Bars represent standard deviations.