NIWA

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Bonamia exitiosus epidemiology in Foveaux Strait oysters

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Objectives 5–10

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Final Research Report

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7. Executive summary

Objectives 5 (to determine the effect of repeated stress on *Bonamia exitiosus*-infected oysters), 6 (to use *in situ* hybridization (ISH) to detect very light *B. exitiosus* infections), 7 (to purify viable *B. exitiosus* from infected oysters), 9 (to determine how long *B. exitiosus* can survive outside the host) and 10 (to determine the distance over which *B. exitiosus* can transmit between oysters) have been completed. The low temperature (8°C) replicate of objective 8 has not been completed due to the need to modify the original experimental design in light of the results obtained from the high temperature (15°C) replicate. Results from Objective 5 indicate that future attempts to maintain heavy *B. exitiosus* infections in oysters in the laboratory should place female or spent oysters at high densities and at high water temperatures (>15°C) under conditions where they are continually disturbed. The results of Objective 6 suggest that heart smears are the most time and cost effective method for screening large numbers of oysters for *B. exitiosus*. In-situ hybridization appears useful for screening small numbers of oysters for *B. exitiosus* in circumstances where high sensitivity is required, while histopathology should be used in epidemiological studies where detection of physiological state, other disease agents or pathological lesions is required. PCR appears to have limited application for detecting *B. exitiosus* infections if not used in conjunction with other diagnostic methods. The results of Objective 7 suggest that a local adaptation of the Percoll density gradient method of Mialhe et al. (1988) is the recommended method to obtain purified *B. exitiosus* for experimental use. The results of Objective 8 experiments conducted at 15°C were used to calculated the 18 week 50% lethal dose of *B. exitiosus* in pre-recruit oysters was around 1.12×10^5 parasites (± 10%). The onset of mortalities of pre-recruit oysters due to bonamiosis appears to be between 3 and 4 months for naïve pre-recruits exposed to environmental levels of *B. exitiosus* via the water, but appears to begin as early as 2.5, 1.5 and 0.25 months after exposure to 10^4, 10^5 and 10^6 *B. exitiosus*, respectively, by injection. The results of Objective 9 suggest that at typical summer water temperatures (18°C), approximately 50% of *B. exitiosus* can survive 48 hours. At typical winter water temperatures (4–10°C), approximately 50% of *B. exitiosus* can survive at least 4 days. The results of Objective 10 suggest that exposure to 10^3 - 10^5 *B. exitiosus* via the water is likely to cause mortalities of 25 to 40% of pre recruit oysters within 18 weeks. These results suggest that the 18 week LD 50 for oysters exposed to *B. exitiosus* via the water is probably only slightly more than that found in Objective 8 for oysters injected with *B. exitiosus*, i.e. around $2 - 3 \times 10^5$ *B. exitiosus*/ oyster. Development of these data to a point where the transmission distance for *B. exitiosus* can be determined requires further information on the number of *B. exitiosus* released when an oyster dies of bonamiosis, and knowledge of the patterns and mechanisms of dispersal of infective particles released from moribund and dead oysters. Further research is recommended to examine the relationship between infection intensity and the actual number of *B. exitiosus*/oyster,

and the *in-vivo* multiplication rate of *B. exitiosus* at various temperatures, to improving the predictive capability of the mathematical model.

8. Overall objective

To gather the information required to develop a mathematical model of the impact and seasonal dynamics of *B. exitiosus* in Foveaux Strait oysters.

Specific objectives

Objective 5. (completed) To determine the effect of repeated stress on *B. exitiosus*-infected oysters.

Objective 6. (completed) To use *in situ* hybridization to detect very light *B. exitiosus* infections.

Objective 7. (completed) To purify viable *B. exitiosus* from infected oysters.

Objective 8. (partially completed) To determine the course of *B. exitiosus* infection from initial infection to death.

Objective 9. (completed) To determine how long the parasite can survive outside the host.

Objective 10. (completed) To determine the distance over which *B. exitiosus* can transmit between oysters.

OBJECTIVE 5:

To determine the effect of repeated stress on *Bonamia exitiosus* infected oysters.

Introduction:

This objective was designed to obtain heavily infected oysters to supply sufficient *B. exitiosus* for the other objectives outlined in this research plan. To obtain a reliable supply of *B. exitiosus*-infected oysters for laboratory experiments, it is usually necessary to amplify the levels of infection of oysters taken from the field.

Method:

A sample of 480 recruit sized oysters (>58 mm shell width) with low level *B. exitiosus* infections (6% prevalence) was collected near the site of the 2001 epizootic in Foveaux Strait. The oysters were divided into 8 groups of 60 oysters. All oysters were scrubbed to remove external fouling organisms. The 8 groups were subjected to the following treatments:

Group 1 (Expose) Sixty oysters were kept on a mesh tray in a large trough (approx 135L) with flow-through seawater. Each day, for 2 weeks, the tray was lifted from the trough and set above the water. Oysters were returned to the tank 8 hours later.

Group 2 (Stir) Sixty oysters were kept in a 50L fibreglass tank with flow-through seawater. Four times a day (approx 0900, 1100, 1400, 1630), for 2 weeks, the oysters were stirred vigorously with a stiff piece of plastic hosing.

Group 3a (Hot) Sixty oysters were kept in a 50L fibreglass tank with flow-through seawater. Each day, for 2 weeks, water flow was stopped for 1 ½ hours. During this time approximately

20L of water was siphoned from the tank and 10L of 75°C seawater was added to the tank. The tank was left for 1 hour at 25-26°C before water flow was recommenced.

Group 3b (Cold) Sixty oysters were kept in a 50L fibreglass tank with flow-through seawater. Each day for 2 weeks water flow was stopped for 1 ½ hours. During this time approximately 20L of water was siphoned from the tank and 4 blocks of frozen seawater were added. The ice was stirred to accelerate cooling. The tank was left for 1 hour after the tank had reached 7°C (usually 10 minutes) before water flow was recommenced.

Group 4a (Hypo-saline) Sixty oysters were kept in a 40L fibreglass tank with aeration. The water was changed daily to maintain acceptable water quality. The salinity was maintained at 15 ‰ for 2 weeks by adding 18L of distilled water to the tank each day. Salinity was checked daily.

Group 4b (Hyper-saline) Sixty oysters were kept in a 40L fibreglass tank with aeration. The water was changed daily to maintain acceptable water quality. The salinity was maintained at 39-40 ‰ for 2 weeks by adding dissolved table salt to the tank each day. Salinity was checked daily.

Group 5 (Filter) Sixty oysters were kept in a 40L fibreglass tank containing aerated seawater filtered to 0.22 µm. Each day for 2 weeks the water in the tank was siphoned out and fully exchanged with fresh 0.22 µm filtered seawater.

Group 6 (Static control) Sixty oysters were kept in a 40L fibreglass tank with aeration. Each day for 2 weeks the water in the tank was replaced with raw seawater.

An additional 100 recruit-sized oysters surplus to the above requirements were maintained in fibreglass troughs as follows:

Group 7 (Trough exposure) Forty five oysters were kept in a mesh bag in a large trough (approx 250L) with flow through seawater. This tank also contained bags of oysters infected with *B. exitiosus*. The treatment oysters were co-habited with the infected oysters for 19 weeks.

Group 8 (Flow control) Fifty five oysters were kept in a large trough (approx 115L) with flow through seawater without any other oysters for 19 weeks.

After their respective 2 week treatments, the oysters in groups 1 to 6 were maintained in flow through aquaria at ambient Wellington Harbour seawater temperatures for another 17 weeks. During this time they and the oysters in groups 7 and 8 were inspected daily and all gapers and dead oysters were removed and fixed for histology in 10% formalin in filtered seawater, and processed using standard histological techniques. Wax sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope to determine the cause of death. After 15 weeks half of the surviving oysters in each treatment were removed and fixed as described above. After 19 weeks all of the surviving oysters were removed and fixed as described above. The mortality rates and intensity of *B. exitiosus* in dead and surviving oysters was then compared between treatments. Water temperature and salinity were monitored daily and ranged between 9–19°C and 32–36 ‰, respectively.

Statistics:

The cumulative mortalities for each treatment were plotted with Kaplan-Meier mortality curves which are designed for censored survival data (Cox 1984).

In this case the censoring was the removal of live oysters at week 15. The mortality at any given week was calculated as:

$$M(t) = 1 - \prod_{\text{weeks} \le t} \left(1 - \frac{\text{no. fatalities during week}}{\text{no. oysters at start of week}} \right)$$

To determine whether significant differences in the prevalence of *B. exitiosus* occurred between treatments and controls after the 4 month observation period, we compared *B. exitiosus* prevalence at 4 months, using a mortality-adjusted prevalence defined as:

$$\frac{\text{number of oysters with } \textit{B. exitiosus}\text{, including those which had already died with } \textit{B. exitiosus}}{\text{original number of oysters, excluding those which had already died without } \textit{B. exitiosus}}$$

Oysters which died but were negative for *B. exitiosus* (including those sampled after 15 weeks) were excluded because they may still have contracted *B. exitiosus* if they had survived the entire 19 weeks. Statistically, this procedure is an approximation to a survival analysis in which oysters dying from causes other than *B. exitiosus* are censored. The statistical significance of the differences in mortality-adjusted prevalence after 19 weeks, between treatments and the pooled static and flow controls, was assessed using a log-linear model:

number of oysters ~ Poisson(mean = Bonamia + sex + treatment + Bonamia*sex + Bonamia*treatment + sex*treatment)

This model also excluded oysters which had already died without contracting *B. exitiosus*.
Treatment effects on the intensity of *B. exitiosus* infections were tested using a linear regression model, using sex as a covariate:

B. exitiosus intensity ~ Normal (sex effect + treatment effect, σ^2).

All oysters, i.e. those sampled alive and those sampled dead, were included. Treatment effects were tested for significance at the $\alpha = 0.05$ level. Sex effects were also examined.

The log-linear models (Agresti 1990) were fitted as a General Linear Model in S-PLUS. The effect of each treatment was tested for significance at the $\alpha = 0.05$ level, using a t-test on the corresponding *B. exitiosus* * treatment interaction. A significant result indicated a statistically significant difference in mortality-adjusted prevalence between that treatment and the pooled controls, allowing for the effect of sex.

The significance of the overall difference between treatments was also tested, using a chi-squared test of residual deviance on the *B. exitiosus**treatment term. A significant result indicated overall significant differences in mortality-adjusted prevalence between treatments, allowing for the effect of sex.

Results:

Mortality: Nearly half of all oysters (49.8%) died during the course of the 19 week experiment (Table 1). The highest three mortality rates were in the hyposalinity, trough exposure and cold treatments, with 100%, 61.3% and 58.3 % mortality, respectively (Figure 1, Table 1). Mortality rates of oysters exposed to hyposalinity (15‰) were 30% after 1 week, 96.6% after 2 weeks and 100% after 3 weeks (Figure 1). However, the prevalence (8.3%) and mean intensity (2.6) of *B. exitiosus* in the oysters which died from the hyposaline treatment were the lowest of all treatments, indicating that mortality was due to the treatment, not *B. exitiosus* infection.

Bonamia prevalence: The treatments which resulted in the highest three prevalences of *B. exitiosus* in both dead and surviving oysters were hot water (96.7%), trough exposure (93.2%), and stir treatments (91.6%) (Table 1). Statistical analysis showed that the prevalence of *B. exitiosus* in oysters exposed to the various individual treatments was not significantly different from that of the controls (Table 2). However, there was a significant ($P = 0.009$) overall difference in *B. exitiosus* prevalence between the treatments themselves. The prevalence of *B. exitiosus* in dead oysters was highest in the hot water treatment (96.3%), followed by the expose (95.8%) and static control treatments (92.9%). The highest prevalence of *B. exitiosus* in surviving oysters was again found in the hot water treatment (97.1%), followed by the trough exposure (94.1%) and stir treatments (92.5%) (Table 1). The lowest prevalence of *B. exitiosus* was in the hyposalinity and filter treatments.

Bonamia intensity: The highest mean intensity of *B. exitiosus* infections in dead oysters was found in the stir treatment (5), followed by the hot water (4.7) and hypersaline treatments (4.6). The highest mean intensity of infections recorded in surviving oysters were from the expose (2.9), stir (2.8) and trough exposure (2.7) treatments (Table 1). Statistical analysis showed that the stir, hot cold, hypersalinity and trough exposure treatments had significantly higher mean *B. exitiosus* intensity than the pooled controls, while the hyposalinity treatment had significantly lower mean *B. exitiosus* intensity (Table 3). The oysters in the filter treatment also had lower intensity *B. exitiosus* infections than controls, but this difference was not significant (Table 3).

Dead vs surviving oysters: The intensity of *B. exitiosus* infections of dead oysters (including the oysters which died in the hyposalinity treatment) was significantly higher (Mann Whitney Rank Sum test, $P < 0.0001$) than those of surviving oysters (Figure 2), suggesting that an increase in intensity of *B. exitiosus* was associated with mortality of the oysters which died during the experiments.

Oyster sex: Female and spent oysters appeared more likely to die than were male and hermaphrodite oysters (Table 1). The regression model found the prevalence of *B. exitiosus* in oysters pooled from all treatments did not vary significantly between sexes (Table 4). However, significant differences in the mean intensity of *B. exitiosus* infections were apparent between sexes, after allowing for the treatment effects (Table 5). The mean intensity of *B. exitiosus* was significantly higher among female and spent oysters than in male and hermaphrodite oysters.

Concurrent infections: A concurrent infection with an undescribed apicomplexan parasite occurred in all oysters examined. However, the intensity of the apicomplexan did not vary significantly between dead (mean intensity 1.9) and surviving oysters (1.9) (Table 1), (Mann Whitney Rank Sum test, $P= 0.956$), indicating that it was not directly associated with mortalities.

Discussion:

Over the duration of these experiments there was a large increase in the prevalence of *B. exitiosus* throughout most treatments and also in control oysters. After 4 months the prevalence of *B. exitiosus* in control oysters (70–80%) and the trough exposure treatment (93.2%) was much higher than the 6% prevalence at the start of the experiments. This suggests that stress due to capture and captive holding of infected oysters at high density was a major factor predisposing oysters to infection with *B. exitiosus*. This is probably due to direct oyster to oyster transmission during cohabitation (Hine 1996). The fact that dead oysters had significantly higher intensity *B. exitiosus* infections than surviving oysters suggests that most of the mortalities observed were due to oysters dying from Bonamiosis, the exception being those oysters in the hyposalinity treatment which appeared to die from osmotic stress.

The prevalence of *B. exitiosus* in treated oysters was not significantly different to that of control oysters, however many of the treatments caused significantly higher intensity *B. exitiosus* infections than were observed in the control oysters. In particular, the increased intensity of *B. exitiosus* in oysters in the trough exposure, stir and hot water treatments were highly significant ($P < 0.001$), suggesting that oysters were additionally stressed by disturbance and warmer waters, that increased oyster density promotes *B. exitiosus* infections, and that *B. exitiosus* probably divides faster at higher temperatures. Rapid proliferation of *B. exitiosus* in naturally infected oysters over summer was discussed by Hine (1991).

Conversely, it appears that exposing oysters to filtered seawater reduces their chances of infection. One of the more notable results was that 15‰ hyposalinity proved lethal to oysters. This is despite evidence that wild *O. chilensis* can tolerate salinities below 10 ‰ in semi enclosed inlets (Buroker et al. 1983, Westerkov 1980). However, in the wild these low salinities are only experienced for short periods of time during the tidal cycle, while in the experiments here oysters were exposed to 15‰ seawater for 2 weeks. This suggests that wild oysters can tolerate quite low salinities for short periods by closing the shell valves and excluding low salinity water, but they require water greater than 15‰ for long term survival. The low prevalence and intensity of *B. exitiosus* in oysters exposed to hyposaline water was similar to that of the oysters sampled prior to the experiments, suggesting that 2 to 3 weeks is insufficient time in captivity to allow an increase in *B. exitiosus* infections to occur.

Hine (1991) showed that oysters in the female stage of the reproductive cycle were more likely to carry heavy infections of *B. exitiosus*, especially during hemocyte resorption of gonads. It appears that *B. exitiosus* utilises lipid from resorbed host eggs for energy, and that spent oysters may exhaust their energy reserves while attempting to fight off infection (Hine and Wesney 1994a,b). The data from the present study supports these observations, as female and spent oysters had significantly higher intensity *B. exitiosus* infections than male and hermaphrodite oysters. Female and spent oysters were also more likely to die from Bonamiosis than were male or hermaphrodite oysters. Spent oysters may have empty follicles as a result of infection reducing the host energy budget causing reduction in, or cessation of, gametogenesis, similar to that reported in *Haplosporidium nelsoni* infections of *Crassostrea virginica* (Barber et al. 1988, Ford & Figueras 1988, Ford et al. 1990

From these data, it is suggested that future attempts to maintain heavy *B. exitiosus* infections in oysters in the laboratory should place female or spent oysters at high densities and at high water temperatures (> 15°C), but at normal salinities, under conditions where they are continually disturbed. This would involve collecting oysters during the late summer and autumn period when the majority of oysters are in the female cycle and natural *B. exitiosus* infections are at their most prevalent (Hine 1991). Once in the laboratory oyster infections could then be maintained throughout the year by keeping water temperatures above 15°C and maintaining high oyster densities by regular replacement of dead oysters when required.

Objective 5: Recommendation:

That future attempts to maintain heavy *B. exitiosus* infections in oysters in the laboratory should place female or spent oysters at high densities and at high water temperatures (>15°C) under conditions where they are continually disturbed.

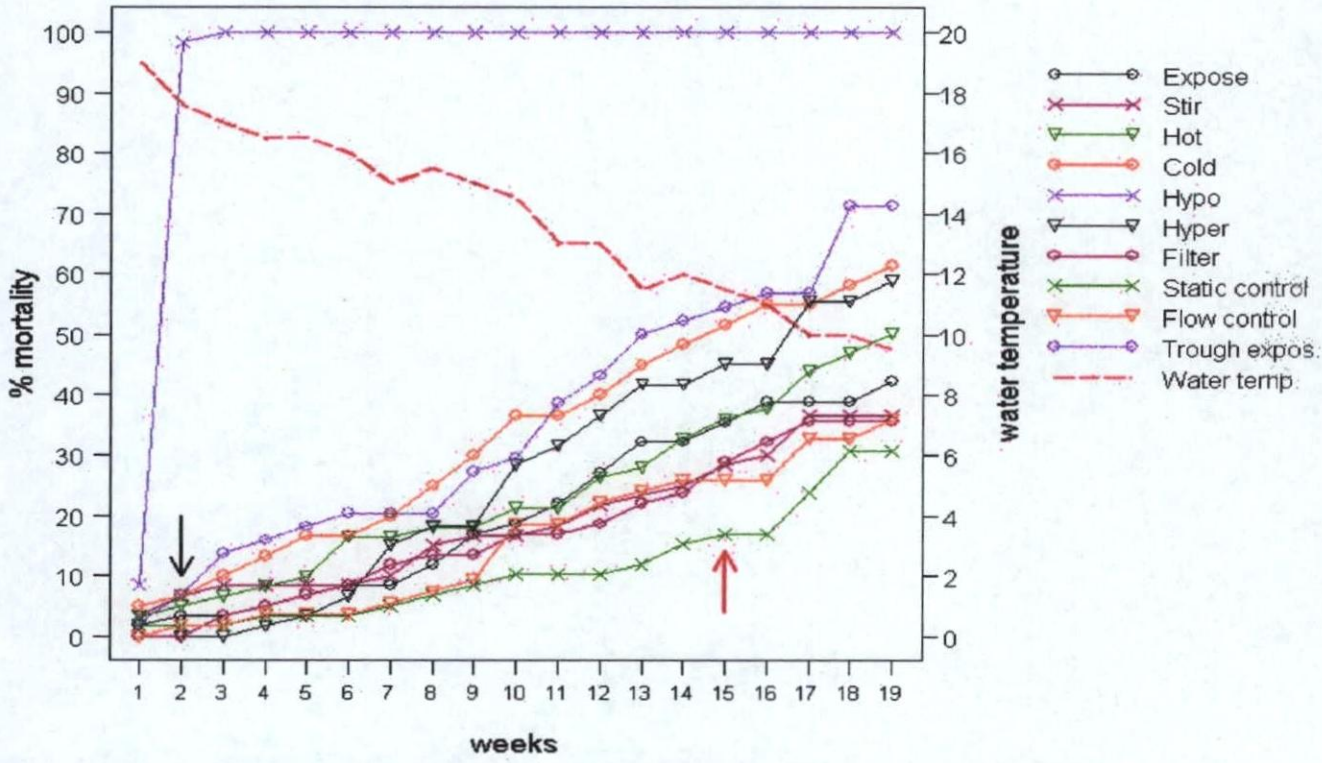

Figure 1. Kaplan-Meier mortality curves for oysters in Objective 5 treatments. Treatments were conducted until the end of the second week (black arrow) and oysters were monitored for 17 weeks post treatment. Half of the surviving oysters were sampled for *B. exitiosus* in week 15 (red arrow).

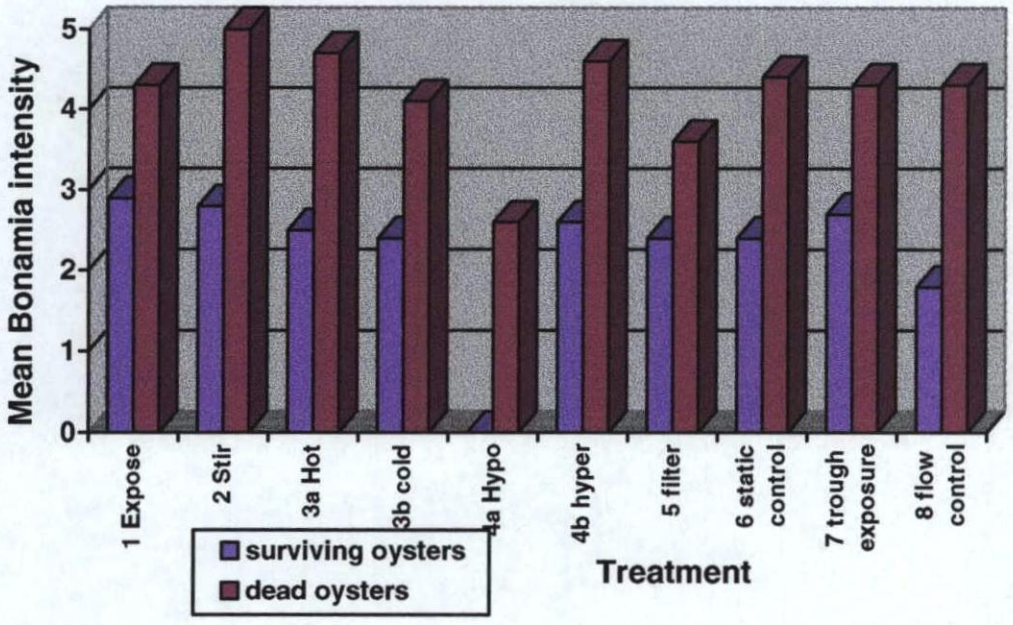

Figure 2. Comparison of the mean intensity of *B. exitiosus* in dead and surviving oysters.

Table 1. Results of Objective 5 experiments after 134 days observation. Figures in bold denote the three treatments with the highest values. * denotes based on a semiquantitative logarithmic scale for which: 0 = not infected, 1 = light infection, 2 = light/moderate infection, 3 = moderate infection, 4 = moderate/heavy infection, 5 = heavy infection.

Treatment	All oysters	Group 1 Expose	Group 2 Stir	Group 3a Hot	Group 3b Cold	Group 4a Hypo	Group 4b Hyper	Group 5 Filter	Group 6 Static control	Group 7 Trough exposure	Group 8 Flow control
No. of oysters	580	60	60	60	60	60	60	60	60	45	55
% male	17.9	22	20	22.9	13.3	10.2	20	22	11.8	13.6	22.6
% female	49.8	50.8	45	52.5	56.6	64.4	51.7	45.7	55.9	29.5	39.6
% hermaphrodite	12.2	11.9	18.3	6.5	11.6	5.1	6.7	13.6	15.3	13.6	20.7
% spent / no sex	20	15.2	16.7	18	18.3	20.3	21.7	18.6	16.9	43.2	17
Total mortality (%)	49.8	40.1	33	44.3	**58.3**	**100**	53.3	33	23.7	**61.3**	33
% mortality male oysters	26.2	**38.5**	16.6	7.2	**37.5**	**100**	33.3	0	28.6	33.3	16.6
% mortality female oysters	57.3	53.3	40.7	56.2	**67.7**	**100**	51.7	48.2	30.3	**84.7**	38.1
% mortality hermaphrodite oysters	14.3	0	**27.3**	0	0	**100**	**25**	0	0	16.6	18.2
% mortality spent/no sex oysters	64.3	33.3	40	72.7	**81.8**	**100**	**84.7**	63.7	20	68.5	55.6
Prevalence *B. exitiosus* – all oysters (%)	75.6	74.6	**91.6**	**96.7**	85	8.3	86.7	64.4	81.4	**93.2**	72.2
Prevalence *B. exitiosus* surviving oysters (%)	79.3	60	**92.5**	**97.1**	76	–	89.3	53.8	77.8	**94.1**	83.3
Prevalence *B. exitiosus* dead oysters (%)	72	**95.8**	90	**96.3**	91.4	8.3	84.4	85	**92.9**	92.6	70.6
Mean intensity *B. exitiosus* live oysters*	2.5	**2.9**	**2.8**	2.5	2.4	–	2.6	2.4	2.4	**2.7**	1.8
Mean intensity *B. exitiosus* dead oysters*	4.3	4.3	**5**	**4.7**	4.1	2.6	**4.6**	3.6	4.4	4.3	4.3
Mean intensity apicomplexan live oysters*	1.9	2	1.5	2	**2.1**	–	**2.2**	**2.1**	1.9	**2.7**	1.6
Mean intensity apicomplexan dead oysters*	1.9	2.2	1.5	1.5	1.8	1	**2.4**	1.8	1.7	**2.5**	**2.4**

Table 2. Results of statistical analyses comparing the prevalence of *B. exitiosus* in oysters from different treatments to that of control oysters. There was no significant difference between treatments and controls, however there was a significant difference ($P < 0.009$) in prevalence between treatments.

Treatment		Mortality-adjusted prevalence after 4 months	P
6, 8	Controls	0.93	–
1	Expose	0.86	0.22
2	Stir	0.98	0.19
3a	Hot	1.00	0.86
3b	Cold	0.93	0.85
4a	Hypo	1.00	0.89
4b	Hyper	0.95	0.70
5	Filter	0.86	0.27
7	Trough exposure	0.98	0.28

Table 3. Results of statistical analyses comparing the mean intensity of *B. exitiosus* in treated oysters compared to that of pooled controls, allowing for the effects of sex.

Treatment		Relative difference from pooled controls in mean *B. exitiosus* intensity	P
6,8	Controls	0	–
1	Expose	0.46	0.08
2	Stir	1.04	<0.001
3a	Hot	1.04	<0.001
3b	Cold	0.58	0.03
4a	Hypo	-2.23	<0.001
4b	Hyper	0.84	0.002
5	Filter	-0.33	0.21
7	Trough exposure	1.24	<0.001

Table 4. Prevalence of *B. exitiosus* after 17 weeks in relation to oyster sex (all treatments pooled).

Sex	Relative mortality adjusted *B. exitiosus* prevalence after 4 months
Male	0.88
Female	0.97
Hermaphrodite	0.89
No sex / spent	0.94

Table 5. Relative intensity of *B. exitiosus* in relation to oyster sex, allowing for treatment effects.

Sex	Relative difference from males in mean *B. exitiosus* intensity	P
Male	0	–
Female	1.14	<0.001
Hermaphrodite	-0.14	0.59
No sex / spent	0.69	0.002

OBJECTIVE 6:

To use *in situ* hybridization to detect very light *Bonamia exitiosus* infections.

Introduction:

Two diagnostic methods are generally used to detect *B. exitiosus* in Foveaux Strait oysters: examination of stained heart imprints (heart smears), and histology. Neither technique is sensitive enough to be sure that oysters are uninfected, and thus a more sensitive technique is needed. The French government marine research agency IFREMER has developed molecular probes specific for the genus *Bonamia* and using these have developed a molecular technique called *in situ* hybridization (ISH). ISH is a powerful technique which uses dye labelled molecular probes to highlight *Bonamia* microcells in tissue sections . Here, the sensitivity of the ISH technique was compared against that of the polymerase chain reaction (PCR). PCR is another molecular technique which uses the same gene probes used in the ISH method, but unlike ISH, PCR does not allow visualisation of the target organism as part of the diagnostic procedure. The PCR methods used here were also not quantitative, in that they provided only a positive or negative result. For this objective the effectiveness of the two molecular techniques were compared against the traditional diagnostic methods of stained heart smears and histology.

Method:

Two groups of oysters were used.

Group 1 oysters: These were sampled from Foveaux Strait in April 2000. Each oyster was firstly used to prepare heart smears (imprints of the heart were made on a microscope slide, air dried then fixed and stained with hemacolor™ before examination under the light microscope). A standard section of each oyster was then fixed in Davidson's fixative for 24 hrs, and then transferred into 70% ethanol for 11 months. Wax blocks were then prepared for a small number of oysters (n = 10) which varied from heavily infected to apparently uninfected, on the basis of the heart smears. Two serial 5 μm thick sections were cut from each of the 10 blocks, one was processed normally for histology and stained with hematoxylin and eosin (H&E), while the other section was processed for ISH following the methods outlined in Appendix 1 (see also Cochennec et al. 2000).

Group 2 oysters: These (n = 60) were freshly shucked over 2 days (13th and 15th of February 2001). They were immediately processed for heart smears, and for oysters collected on 15/2/01 small pieces of gonad and digestive gland were fixed in 70% ethanol for PCR. A standard section of oyster was then fixed for no more than 48 hours in either 10% formalin in filtered seawater, or Davidson's fixative before being embedded in wax and processed for histology and ISH as for group 1 oysters. The PCR procedures and primers used amplified a 350 bp region of *Bonamia* 18S rDNA (Cochennec et al. 2000) as outlined for preparation of the labeled ISH probe (Appendix 1). The relative sensitivities of the 4 diagnostic techniques were then compared. Positive controls (European flat oysters *Ostrea edulis* infected with *B. ostreae*) were included in every batch of slides in the ISH method and every set of PCR reactions to ensure that false negatives were not due to poor sample preparation. PCR results were graded as either positive or negative. The intensity of *B. exitiosus* infections detected by heart smears, histology and ISH was scored using semiquantitative techniques (Table 6).

Test results were evaluated using standard epidemiological methods (after Fegan 2000, see Appendix 2) for:

- sensitivity (proportion of oysters with *B. exitiosus* which test positive – proportion of true positives),
- specificity (proportion of oysters without *B. exitiosus* which test negative – proportion of true negatives),
- positive predictive value (PPV, the probability that an oyster returning a positive test actually has *B. exitiosus*), and
- negative predictive value (NPV – the probability that an oyster which returns a negative test actually does not have *B. exitiosus*).

The kappa statistic value of each diagnostic method, compared against a "gold standard" of the pooled results of all tests combined, was also calculated to compare the relative effectiveness of the respective treatments for detecting *B. exitiosus*. The Kappa values were evaluated according to the arbitrary benchmarks in Table 7.

Results:

Group 1 oysters
A comparison of the sensitivity of heart smears, histology and ISH was done on 10 group 1 oysters (Table 8). While 50% of these oysters were positive for *B. exitiosus* by heart smears, and 30% by histology, none were positive for *B. exitiosus* using the ISH method.

Group 2 oysters
In the sample of 30 oysters collected on 13/2/01, the "true" prevalence of *B. exitiosus* found when the results of all methods used was pooled was 60% (Table 9). The method which recorded the highest apparent prevalence of *B. exitiosus* was hearts smears, which recorded a 56.7% apparent prevalence at a mean intensity of 3.2. The only false negative recorded for the heart smear method was in an oyster which was categorised as 2 using histology (Table 9). Histology found an apparent prevalence of 50% at a mean intensity of 3.1. Histology produced 3 false negatives (10% incidence), all of which were light (grade 1) infections picked up by heart smears. *In-situ* hybridisation of formalin fixed oysters also recorded 50% apparent prevalence, due to two false negatives (one grade 1 infection detected by heart smear, and one grade 2 infection detected by histology), and 3 "not possibles" (10 %) where the sections had detached from the microscope slides. Of these 3 not possibles, one was an infected oyster, hence the number of false negatives for ISH for this sample was 3 (10 %) (Table 9).

The 30 oysters collected on 15/2/01 had a "true" *B. exitiosus* prevalence of 96.7% when the results of all methods used were pooled (Table 9). In this group ISH of formalin fixed oysters produced the highest apparent prevalence of 83.3% with 4 false negatives, these being 2 "not possibles" and 2 oysters positive by PCR. PCR recorded an apparent prevalence of 76.7%, including positives for oysters 21 and 22, which both tested negative using all other techniques. There were 6 false negatives (20% incidence) recorded using PCR, mostly in oysters which showed light infections by ISH and heart smears. Heart smears recorded the next highest apparent prevalence of 60%, but there were 9 false negatives using this method on lightly infected oysters which were diagnosed using ISH of formalin fixed material and 2 more for oysters 21 and 22 which were positive by PCR. ISH of Davidson's fixed material recorded an apparent prevalence of 50%, with most false negatives due to 11 "not possibles" on infected oysters as well as 3 negatives on lightly infected oysters (score 1–2) diagnosed by ISH and in one case, by heart smear. Histology provided the lowest apparent prevalence of only 43.3%, with 16 false negatives (53.3%) on light to moderately infected oysters (score 1–3) which were detected by ISH, PCR and heart smears (Table 9).

The results for the oysters collected on the 13/2/01 and 15/2/01 were then pooled to allow comparison of these diagnostic methods against a "gold standard" of the combined results of all tests (78.3% prevalence of *B. exitiosus* in the 60 group two oysters examined. For the first analysis (Table 10a), "not possible" results due to loss of sections were classed as negative results, and it was assumed that no method gave false positives, giving all tests 100% specificity and PPV. Using these criteria the test with the highest sensitivity (85.1%) and NPV (65) was ISH of formalin fixed oysters, which recorded an apparent prevalence of 66.7%. PCR had a sensitivity of 79.3% with a NPV of 14.3 and apparent prevalence of 76.7%. The true prevalence for the 30 oysters sub sampled by PCR sample was 96.7%. The next most sensitive test was heart smears (74.5 % sensitivity, 52 NPV, apparent prevalence 58.3%), followed by histology (59.6% sensitivity, 40.6 NPV, 46.7% apparent prevalence). The kappa value for heart smear results (0.56) indicated that they gave moderate agreement with the results of all tests combined, while histology only gave fair agreement (kappa value 0.39). The least sensitive test was ISH on Davidson's fixed oysters (51.7 % sensitivity), mainly due to the high number of "not possibles" due to loss of sections from slides. The latter method had the lowest NPV (6.7) and kappa values (0.07) of all the tests examined, indicating poor agreement with the results of all tests combined (Table 10a).

A second analysis was then undertaken assuming that the PCR positives on oysters 21 and 22 sampled on 15/2/01 (which tested negative using all other techniques), were false positives. Also for oysters examined by ISH the data were analysed both including, then excluding, "not possible" results. This analysis (Table 10b) again found that the most specific method was ISH of formalin fixed material. For this method specificity was 88.9% when "not possibles" were included, and 95.2 % when the 5 "not possibles" were excluded from the data set. This method also had the highest NPV of 75 (86.7). The kappa value for ISH of formalin fixed material was 0.8, signifying a substantial agreement with the benchmark tests, and provided an almost perfect agreement (kappa = 0.9) when the "not possibles" were excluded. Specificity and PPV were 100% due to the need to visualise *B. exitiosus* microcells which were positive to the ISH probe to achieve a positive result.

Heart smears and PCR were equally the next most sensitive tests at 77.8 % sensitivity (Table 10b). Because it was assumed PCR gave 2 false positives, specificity (33.3%) and PPV (91.3%) were relatively low for this method relative to heart smears, where the requirement of visualisation of *B. exitiosus* microcells to achieve a positive result meant that specificity and PPV were 100%. Heart smears also had a much better NPV (60 compared to 14.3 for PCR), and hence achieved a much better kappa evaluation (0.64, substantial agreement with benchmark tests) than did PCR (kappa value 0.07, poor agreement with benchmark tests).

ISH of Davidson's fixed material had a high sensitivity (83.3%) only when all "not possibles" were excluded, which limited sample size for this method to only 18 oysters. All of these 18 oysters were positive for *B. exitiosus* using other tests, so it was not possible to calculate specificity, NPV or kappa values when the "not possibles" were excluded (Table 10b). In fact, the method achieved better specificity (100%), NPV (20) and kappa value (0.2) when "not possibles" were included as negative results, even though sensitivity was lower (55.6%) when this was done.

Histology achieved a moderate sensitivity of 62.2 %, but high specificity (100%) and PPV (100%) due to the need to visualise *B. exitiosus* microcells in sections to achieve a positive result. The NPV for histology (46.9) was the third highest of all methods and the kappa value of 0.45 indicated moderate agreement with the benchmark results of all tests combined.

Discussion:

The results with the group 1 oysters indicated that the ISH method could be unreliable and was likely to provide false negative results if oyster samples were not fixed or stored in a suitable manner. The accepted sample preparation methods used to provide optimal results for ISH are fixation in Davidson's fixative for no longer than 48 hours, then prompt embedding of samples into wax (N. Cochennec–Laureau, IFREMER, and G. Burreson, VIMS, pers. communication). Our data indicate that prolonged (11 month) storage of samples in ethanol, which theoretically should result in acceptable preservation of *B. exitiosus* DNA, nevertheless did not preserve oyster tissues in a condition suitable for reliable ISH results.

In contrast, group 2 oyster samples that were fixed in 10% formalin for no more than 48 hours, then immediately processed and embedded in wax, provided excellent results with ISH. Under these conditions ISH of formalin fixed sections was more sensitive for detecting *B. exitiosus* than any other method, and also had the highest chance of detecting true negatives (NPV). PCR was the second most sensitive method examined, followed by examination of heart smears, then histology. ISH of Davidson's fixed material was the least sensitive method. However, the lack of sensitivity for ISH of Davidson's fixed oysters was mostly due to numerous slides where "not possible" results were obtained, rather than failure of the probe to hybridise to sections. Indeed the major technical drawback observed with the ISH method during these experiments was partial or total loss of sections from slides during ISH processing (causing "not possible" results). However we subsequently found this error could be minimised by using plastic coverslips (Hybaid easi seal type) and slides coated manually with APES (3–Aminopropyltriethoxysilane). Once these modifications were incorporated into the ISH methodology, good section retention and more sensitive results could be obtained (i.e. the ISH results for the formalin fixed oysters).

The specificity and PPV of heart smears, histology and ISH was assumed to be 100%, due to the requirement to visualise *B. exitiosus* microcells in sections and smears to achieve a positive result with these tests. Lack of specificity was considered a major issue only with the PCR method, where a positive result does not require visualisation of the parasite. It is known that false PCR positives can occur when standardisation and validation of PCR results is not performed, even under stringent laboratory conditions (Walker and Subasinghe 2000). This is why a PCR positive result by itself is usually not considered enough to confirm the presence of a pathogen. Usually PCR results must be supported by other diagnostic methods before a confirmatory diagnosis is obtained (Walker and Subasinghe 2000). Because oysters 21 and 22 collected on 15/2/01 were positive for *B. exitiosus* only using PCR, but were negative using all other tests, with the data currently available it is impossible to rule out that these were false PCR positives. If it was assumed these were false positives, PCR provided the lowest specificity, PPV, NPV and kappa values of all of the methods examined. Even when no false positives were assumed for PCR, this method generated the second lowest NPV and kappa values of all tests examined. These data indicate that PCR has limited application for detecting *B. exitiosus* infections if not used in conjunction with other diagnostic methods.

False PCR positives usually arise from the exquisite sensitivity of the method, however in the present comparison the sensitivity of PCR was second to ISH. Therefore, ISH of formalin fixed material must be the recommended diagnostic tool for detecting light *B. exitiosus* infections due to its high sensitivity, specificity, negative predictive value and the fact that ISH positive *B. exitiosus* microcells can actually be visualised in sections. However, specificity could be a minor issue with the ISH method. Not only does the ISH probe used here hybridise with both *B. ostreae* from France and *B. exitiosis* from New Zealand, in other laboratory trials we noted the probe also hybridised with other haplosporidians, namely a haplosporidian parasite of paua

(*Haliotis iris*) (data not published). This indicates there may be a need to develop other primer sets to produce a more specific probe if the ISH method was used in areas where mixed infections of *Bonamia* and other haplosporidian species were suspected to be present. These observations do not detract from the usefulness of the existing ISH probe in New Zealand flat oysters, however, as *B. exitiosis* is the only haplosporidian known from this species.

The other drawback which became apparent when using the ISH method was its expense (around $30 NZD per oyster, compared to $15 for histology and PCR, and $9.50 for heart smears). Much of the expense was associated with the extra time needed to process each sample due to the numerous manipulations and steps required for the ISH process, and the cost of the expensive molecular reagents required to make the probes. Because of the high cost of ISH, heart smears were considered the diagnostic method representing the best compromise between sensitivity, specificity, NPV and cost effectiveness. Examination of heart smears was not only the equal second most sensitive method for detecting *B. exitiosus* in the oysters examined, it was by far the fastest and also the least expensive. These attributes point to heart smears being the most cost effective method for screening large numbers of oysters for *B. exitiosus* (such as in large scale surveys in Foveaux Strait).

ISH appears very useful for screening smaller numbers of oysters for *B. exitiosus* in circumstances where high sensitivity is required (e.g. screening for *B. exitiosus* free populations of oysters, developing a "gold standard" diagnostic technique), provided good control of sample fixation, rapid sample processing, the necessary laboratory equipment, and sufficient funds are available. We found that fixation for no more than 48 hours in 10% formalin in seawater provided the most sensitive results with ISH, indicating that choice of fixatives for ISH of *B. exitiosus* is not limited solely to Davidson's fixative. Longer than 48 hours fixation of oysters in either fixative, or transfer of oysters to 70% ethanol for prolonged periods of time, is not recommended, as the chances of false negative results with ISH may increase to unacceptable levels. Immediate embedding of fixed oysters into wax blocks and prompt processing and sectioning of these will promote the best conditions for ISH. Nevertheless, there is anecdotal evidence for good long term retention of DNA in wax embedded material, and the storage life of wax embedded material should be determined. The possibility of generating false negatives associated with loss of sections from slides during the ISH procedure should also be considered. However, incorporation of plastic "eziseal" coverslips and slides coated manually with APES (rather than use of proprietary "silane–prep" slides) into the ISH methodology resulted in good section retention and minimal "not possible" results, hence these particular steps are recommended in order to maximise the sensitivity of ISH.

Histology provided the second lowest sensitivity in these experiments, but still remains a very important diagnostic method because of the ability to detect other disease agents (such as the apicomplexan, bacteria and parasites), pathological lesions (e.g. hemocytosis, necrosis) and morphological features (gonad condition etc.) which can be used to provide important information on the overall condition of each oyster. For these reasons, histology is an indispensable technique which should be used wherever possible to provide important information, such as potential predisposing factors in Bonamiosis (concurrent infections, sex, state of maturation etc.) so that the epidemiology of the disease can be better understood.

Objective 6: Recommendation:

Heart smears are the most time and cost effective method for screening large numbers of oysters for *B. exitiosus*. ISH appears useful for screening smaller numbers of oysters for *B. exitiosus* in circumstances where high sensitivity and specificity is required, while histopathology should be used in epidemiological studies where detection of physiological state, other disease agents or pathological lesions is required. PCR has limited application for detecting *B. exitiosus* infections if not used in conjunction with other diagnostic methods.

Table 6. Semiquantitative scoring techniques used to grade *B. exitiosus* infections in heart smears, histology and *in-situ* hybridisation (ISH).

Grade	Heart smears	Histology and ISH
0	Not infected	Not infected
1	One *B. exitiosus* observed after examining an imprint	Very few (<10) *B. exitiosus* observed after extensive searching of all tissues
2	More than 1, but less than 10 *B. exitiosus* in an imprint	*B. exitiosus* observed only after searching, and then only one or two present in each infected hemocyte
3	More than 10 *B. exitiosus* per imprint, but few parasites per hemocyte	*B. exitiosus* widespread, but only 1 to 5 per hemocyte
4	*B. exitiosus* present in many hemocytes of each imprint, and many parasites in each hemocyte	*B. exitiosus* readily observed in all tissues, often associated with hemocytosis
5	*B. exitiosus* present in nearly all hemocytes in each imprint, many parasites in each hemocyte and extracellularly.	*B. exitiosus* abundant in all tissues, many per hemocyte and often extracellular. Hemocytosis always present, lesions sometimes observed

Table 7. Arbitrary benchmarks used to evaluate Kappa values for diagnostic tests (Fegan 2000).

Kappa value	**Evaluation**
>0.81	Almost perfect agreement
0.61–0.80	Substantial agreement
0.41–0.60	Moderate agreement
0.21–0.40	Fair agreement
0.01–0.20	Slight agreement
0.00	Poor agreement

Table 8. Comparison of the relative scores for heart smears, histology and *in-situ* hybridisation (ISH) for detecting *B. exitiosus* infections in group 1 Foveaux Strait oysters after 24 hours fixation in Davidson's fixative then storage in ethanol for 11 months. Red = false negative. * based on a semiquantitative scoring techniques described in table 6.

Oyster number	**Heart smear**	**H&E section**	***In-situ* hybridisation (ISH Davidson's)**
1/2	4	0	0
1/3	0	0	0
1/4	2	3	0
1/5	2	3	0
1/6	2	2	0
1/7	3	0	0
1/9	0	0	0
1/11	0	0	0
1/13	0	0	0
1/15	0	0	0
Prevalence	**50 %**	30%	0%
Mean Intensity*	2.6	2.7	–
False negatives	0%	20%	**50%**

Table 9. Comparison of the relative scores for heart smears, histology and *in-situ* hybridisation for detecting *B. exitiosus* infections in fresh samples of Foveaux Strait oysters (group 2 oysters). Red = false negative. NP = not possible due to loss of section during processing. * based on semiquantitative scoring techniques described in methods.

	Fresh samples 13/2/01				Fresh samples 15/2/01					
Oyster no.	**Heart smear**	**H& E section**	**ISH formalin**	**All methods**	**Heart smear**	**H&E section**	**ISH Davidson**	**ISH formalin**	**PCR**	**All methods**
1	0	0	NP	**0**	2	0	0	1	1	**1–2**
2	0	0	0	**0**	1	0	1	2	0	**1–2**
3	2–3	3	2	**2–3**	0	0	0	1	1	**1**
4	1	0	1	**1**	5	3–4	3	5	1	**3–5**
5	4–5	3	2	**2–5**	4	3–4	3	4	1	**3–4**
6	5	3–4	4	**3–5**	3	3	NP	1	1	**1–3**
7	2	3–4	3	**2–4**	0	0	1	1	1	**1**
8	3–4	3–4	4	**3–4**	0	1–2	1	1	1	**1–2**
9	4	3	2	**2–4**	0	0	NP	0	0	**0**
10	0	0	0	**0**	3	3–4	4	3–4	1	**3–4**
11	0	2	0	**2**	4	3–4	4	3	1	**3–4**
12	5	4	4	**4–5**	3	0	1	NP	0	**1–3**
13	3–4	3	1	**1–4**	3	2–3	1	NP	1	**1–3**
14	4	3–4	NP	**3–4**	3	0	1	2	1	**1–3**
15	0	0	0	**0**	0	0	NP	1	1	**1**
16	1	0	0	**1**	3	3	2–3	2–3	1	**2–3**
17	0	0	0	**0**	4	3–4	4	3–4	1	**3–4**
18	0	0	0	**0**	0	0	NP	3	0	**3**
19	4	3	1	**1–4**	0	0	0	2	1	**2**
20	0	0	0	**0**	0	0	NP	1	1	**1**
21	3–4	3	3	**3–4**	0	0	NP	0	1	**1**
22	0	0	0	**0**	0	0	NP	0	1	**1**
23	2–3	3	2	**2–3**	1	3	3–4	3	1	**1–3**
24	0	0	0	**0**	0	0	NP	1	0	**1**
25	0	0	0	**0**	0	0	NP	1	0	**1**
26	1	0	1	**1**	1–2	0	NP	1	0	**1–2**
27	3	2	1	**1–3**	4	3–4	2–3	3	1	**2–4**
28	0	0	0	**0**	3	0	NP	3	1	**3**
29	5	4	4	**4–5**	5	2–3	2	4–5	1	**2–5**
30	0	0	NP	**0**	3	2–3	NP	2	1	**2–3**
No. oysters infected	**17**	15	15	**18**	18	13	15	**25**	**23**	**29**
Prevalence	**56.7%**	50%	50%	**60%**	60%	43.3%	50%	**83.3%**	76.7%	**96.7%**
Mean Intensity*	**3.2**	3.1	2.3	–	**3.1**	3.0	2.3	2.2	–	–
False negatives	3.3%	**10%**	**10%**	–	36.7%	**53.3%**	46.7%	13.3%	20%	–
Not possible	0%	0%	**10%**	–	0%	0%	**40%**	6.7%	0%	–
Prevalence (not possible results excluded)	**56.7%**	50%	55.6%	60%	60%	43.3%	83.3%	**89.3%**	76.7%	**96.7%**

Table 10a. Evaluation of diagnostic tests used to detect *B. exitiosus* in group 2 Foveaux Strait oysters, assuming no false positive results occurred and considering all "not possible" results as negatives. ISH of formalin fixed oysters provided results which were in substantial agreement to the results of all tests combined. In the absence of false positives, specificity and PPV for all tests is by definition 100%.

	Heart Smears	Histology	ISH formalin fixed	ISH Davidson's fixed	PCR	All tests combined
No. oysters examined	60	60	60	30	30	60
Sensitivity (%)	74.5	59.6	85.1	51.7	79.3	100
Negative predictive value	52	40.6	65	6.7	14.3	100
Apparent prevalence	58.33%	46.7%	66.7%	50%	76.7%	78.3%
True prevalence	78.3%	78.3	78.3%	96.7%	96.7%	78.3%
Kappa value	0.56	0.39	0.71	0.07	0.2	1
Kappa evaluation (agreement with benchmark)	Moderate	Fair	Substantial	Slight	Slight	Benchmark

Table 10b. Evaluation of diagnostic tests used to detect *B. exitiosus* in group 2 Foveaux Strait oysters, assuming 2 false positive results were generated by PCR. Figures in parentheses indicate ISH results when all "not possibles" were excluded. ISH of formalin fixed oysters provided results which were in substantial agreement with the benchmark results of all tests combined. Heart smears were also in substantial agreement to the results of all tests combined.

	Heart Smears	Histology	ISH formalin fixed	ISH Davidson's fixed	PCR	All tests combined
No. oysters examined	60	60	60 (55)	30 (18)	30	60
Sensitivity (%)	77.8	62.2	88.9 (95.2)	55.6 (83.3)	77.8	100
Specificity (%)	100	100	100 (100)	100 (n/a)	33.3	100
Positive predictive value (PPV)	100	100	100 (100)	100 (100)	91.3	100
Negative predictive value (NPV)	60	46.9	75 (86.7)	20 (0)	14.3	100
Apparent prevalence	58.33%	46.7%	66.7% (72.7%)	50% (83.3%)	76.7%	75%
True prevalence	75%	75%	75% (76.4%)	90% (100%)	90%	75%
Kappa value	0.64	0.45	0.8 (0.9)	0.2 (0)	0.07	1
Kappa evaluation (agreement with benchmark)	Substantial	Moderate	Substantial (Almost perfect)	Slight (Poor)	Poor	Benchmark

OBJECTIVE 7:

To purify viable *Bonamia exitiosus* from infected oysters.

Introduction:

In order to carry out studies on the survival of *B. exitiosus* outside the host, transmission in relation to distance, and the course of infection, it is necessary to have quantified viable purified *B. exitiosus*. Purification techniques have been developed for *B. ostreae* (see Mialhe et al. 1988), and a similar sized pathogen of oysters, *Mikrocytos roughleyi* (see Hervio et al. 1996), using sucrose density gradient centrifugation. Purification of *B. ostreae* showed the yield to be between 10^7 and 5 x 10^7 per heavily infected oyster (Mialhe et al. 1988).

Method:

Foveaux Strait oysters were shucked 10 or 20 at a time, heart imprints prepared and stained, and the imprints examined microscopically for the presence of *B. exitiosus*. Heavily infected oysters were then homogenised, and layered over sucrose and percoll density gradients following the methods described by Mialhe et al. (1988). After centrifugation, the cells at each interface were removed, quantified using a haemocytometer, and subsamples were stained with trypan blue and examined under a light microscope, to assess viability. The level of microbial contamination was also assessed qualitatively by microscopy. The levels of *B. exitiosus* retrieved were recorded and modifications of various steps of the protocol were made in attempts to improve the yield and viability of purified *B. exitiosus*.

Results and discussion:

After numerous attempts at purification using the methods of Mialhe et al. (1988), a revised purification protocol was devised so that between 10^6 and 10^7 purified viable *B. exitiosus* could be reliably obtained from 2 or 3 moderately to heavily infected oysters, with little microbial contamination. The revised protocol is reproduced in full in Appendix 3. Also during the course of this work a new method for purifying *Mikrocytos mackini*, causative agent of Denman Island disease in Pacific oysters (*Crassostrea gigas*) in Canada, was published (Joly et al. 2001). A modification of this simple filtration technique was trialled to determine whether the technique could be adapted for purification of *B. exitiosus*. However, *B. exitiosus* was not successfully purified using the filtration technique. This indicates that while simple filtration techniques may work for the smaller (2–3μm) *Mikrocytos*, they are less successful for *B. exitiosus* which requires a larger filter pore size due to its larger size (4–5 μm). The larger pore size filters tended to allow bacteria and many host cells through, contaminating the filtrate.

Objective 7: Recommendation:

The local adaptation of the Percoll density gradient method of Mialhe et al. (1988) is the recommended method to obtain purified *B. exitiosus* for experimental use.

OBJECTIVE 8:

To determine the course of *Bonamia exitiosus* infection from initial infection to death.

Introduction:

Information on the course of *Bonamia exitiosus* infection is required for direct application to statistical models, and in order to maintain a supply of heavily infected oysters in the laboratory. Originally it was planned that the experiments were to be carried out at 8°C and 15°C, which is the temperature range on the bottom in Foveaux Strait. At present results are available for the oysters injected and held at 15°C.

Method:

Source of oysters

Groups of 20 recruited (>58 mm width) wild- caught oysters and 10 - 20 pre–recruit (<58 mm width) cultured dredge oysters were each held in small aquaria (40 x 20 x 20 cm) at 15°C water temperature. The recruited oysters were obtained in March 2001 from a station in Foveaux Strait which had a low prevalence of *B. exitiosus* infections (Diggles and Hine, unpublished data) and were maintained in captivity at Greta Point for 4 weeks prior to the commencement of the experiment in May 2001. The pre–recruit oysters were used immediately after being obtained from a commercial supplier from the Marlborough Sounds in August 2001, after finding no evidence of *B. exitiosus* during sampling of 60 oysters from that supplier by histology and heart smears. These data indicated that, if *B. exitiosus* was present in the pre-recruit oysters, there was 95% confidence it was present at a prevalence of less than 5%, assuming the diagnostic methods used were 100% effective for detecting the presence of *B. exitiosus*.

Experimental infections

Each oyster was removed from the water and a needle access hole was drilled through the shell with a 6 mm drill bit. Sterile, 25 mm long, 25 gauge needles were used to inoculate either 100 µl or 200µl of a suspension containing either 10^2, 10^4, or 10^5 purified viable *B. exitiosus* in 0.22 µm filtered seawater (FSW) into the digestive gland (Table 11). A smaller number (n = 15) of pre–recruit oysters were also injected with 10^6 purified viable *B. exitiosus* in 100 to 300 µl of 0.22 µm FSW (Table 11). Groups of 20 recruited and pre–recruit oysters were also drilled and injected with 100 to 300 µl of FSW as controls (Table 11). Each oyster was kept out of the water for 5 minutes after injection before being placed back into their respective tanks. All oysters were then monitored for up to 20 weeks. During this time they were inspected daily and all gapers and dead oysters were removed and fixed for histology in 10% formalin in filtered seawater, and processed using standard histological techniques. Wax sections were stained with hematoxylin and eosin (H&E) and examined with the light microscope to determine the cause of death. After 20 weeks for recruited oysters, and 18 weeks for pre-recruits, all surviving oysters were fixed as described above and examined for the presence of *B. exitiosus* using routine histology. Infections were graded using the following semiquantitative scoring method:

- Grade 0, not infected;
- Grade 1; very few (<10) *B. exitiosus* observed after extensive searching of all tissues;
- Grade 2; *B. exitiosus* observed only after searching, and then only one or two present in each infected haemocyte;
- Grade 3; *B. exitiosus* widespread, but only 1 to 5 per haemocyte;

- Grade 4; *B. exitiosus* readily observed in all tissues, often associated with haemocytosis; and Grade 5; *B. exitiosus* abundant in all tissues, many per haemocyte and often extracellular. Haemocytosis always present, lesions sometimes observed.

Oysters with grade 1 or 2 infections were classified as having light *B. exitiosus* infections. Oysters with grade 3 infections were classified as having moderate *B. exitiosus* infections, while those with grade 4 and 5 infections were classified as having heavy *B. exitiosus* infections.

Results:

Recruited oysters

High mortality rates of controls in the experiment using recruited oysters (Figure 3) strongly indicated that the oysters used had existing *B. exitiosus* infections prior to the beginning of the experiment. After 20 weeks, mortality rates were 100% for all treatments and 80% for controls. The prevalence of *B. exitiosus* infections in controls was 100% (Figure 4), and control oysters with heavy *B. exitiosus* infections began to die after 3 weeks in the experimental tanks (Figure 5). A similar trend was observed in the treatment tanks where heavily infected oysters began to die within the first 2 or 3 weeks of the experiment (Figures 6-8). The presumption that the mortalities observed in both treatment and control recruited oysters was due to bonamiosis was supported by the high mean intensities of *B. exitiosus* recorded in both control and treatment oysters (Figure 9).

The prevalence and intensity of an unnamed coccidian parasite (Figures 4, 9) was relatively low compared to *B. exitiosus*, suggesting the presence of the former did not directly cause the mortalities observed.

Pre-recruit oysters

Bonamia prevalence: The prevalence of *B. exitiosus* in the oysters in the 10^6 treatment was 100% (Figure 11). The prevalence of *B. exitiosus* in oysters in the 10^2,10^4 and 10^5 treatments ranged between 80 and 90%, while the prevalence of *B. exitiosus* in control oysters was 60% (Figure 11).

Mortalities: The highest mortality rate of 80% was recorded for oysters injected with 10^6 *B. exitiosus* (Figure 10). The second highest mortality rate recorded was for those in the 10^5 treatment (45% mortality), followed by control oysters (40% mortality), and oysters in the 10^4 and 10^2 treatments (30% and 10% mortality, respectively) (Figures 10, 12-16). Mortalities in control pre-recruit oysters began in the 10^{th} week of when an oyster with a moderate *B. exitiosus* infection died (Figure 12). Two uninfected control oysters died in the 12^{th} week while in the 15^{th} week another uninfected oyster died together with two heavily infected oysters. The remaining two mortalities in control oysters occurred in the 17^{th} week when an uninfected oyster died along with a heavily infected oyster (Figure 12).

Two pre-recruit oysters injected with 10^2 *B. exitiosus* died in the 9^{th} week of the experiment, both with moderate *B. exitiosus* infections (Figure 13). Oysters injected with 10^4 *B. exitiosus* began to die in the 5^{th} week of the experiment and 30% had died by the beginning of the 15^{th} week, all with moderate *B. exitiosus* infections (Figure 14). One oyster injected with 10^5 *B. exitiosus* died with heavy *B. exitiosus* infections in the 4^{th} week post injection (Figure 15). Moderately infected oysters died in the 10^{th} and 14^{th} week while heavily infected oysters died in the 15^{th} and 17^{th} weeks. Two oysters injected with 10^5 *B. exitiosus* died in the 16^{th} week but *B. exitiosus* was not detectable by histopathology in these oysters.

Pre-recruit oysters injected with 10^6 *B. exitiosus* began dying in the first week post exposure, and continued to die at regular intervals throughout the experiment (Figure 16). All oysters in this treatment had *B. exitiosus* infections detectable by histopathology, with the majority of mortalities at the later stages being associated with heavy infections (Figure 16).

Bonamia intensity: The treatment which resulted in the heaviest mean intensities of *B. exitiosus* infections (for both dead and surviving oysters) was the 10^5 treatment (mean intensity 4), followed by the 10^6 treatment (3.9), the 10^2 treatment, (3.1), and the 10^4 treatment and controls (mean intensity 2.9) (Figure 17). The prevalence and intensity of an unnamed coccidian parasite (Figures 11, 17) was relatively low compared to *B. exitiosus*, suggesting the presence of the former did not directly cause the mortalities observed.

Lethal dose: The 18 week 50% lethal dose was calculated to be 111,200 *B. exitiosus* (Table 12) using the method of Reed and Muench (1938).

Discussion:

Recruited oysters: The first experiment used recruited oysters from Foveaux Strait. Their mortality rates strongly suggested that most of the oysters used had pre-existing *B. exitiosus* infections prior to the experimental inoculations. The prevalence of *B. exitiosus* in the oysters used in the experiments approached 100% and virtually all of the oysters which died, including control oysters, had heavy *B. exitiosus* infections, even the oysters which died as early as the second or third week of the experiment. These results contrast those of Hervio et al. (1995), who found that most European flat oysters (*Ostrea edulis*) experimentally infected with an 100% infective dose of *Bonamia ostreae* died of bonamiosis no earlier than 2 months after infection, and that most mortalities occurred after 3 or 4 months at 18°C. Furthermore, European field data indicates that in natural situations, *B. ostreae* becomes detectable 3 - 4 months after introducing uninfected oysters into areas where *B. ostreae* is enzootic (Tige and Grizel 1984, Montes 1991). Our results thus suggest that while the oysters sampled from Foveaux Strait in March 2001 may have had low or undetectable infections at the time they were collected, by the time they were used for the experiment, (after 2 months in captivity at Greta Point), a significant increase in *B. exitiosus* prevalence and/or intensity had occurred. The data obtained on the course of *B. exitiosus* infection from the experiment which used recruit sized oysters must, therefore, be considered unreliable.

The results of the experiment with wild caught recruit sized oysters highlighted the need to locate a source of oysters which were preferably naïve to *B. exitiosus*, or else had undetectable infections at the beginning of the experiments. At the time these experiments were conducted, it was impossible to guarantee that a sample of 100 wild oysters from Foveaux Strait would be free from *B. exitiosus* infection, such was the extent of the *B. exitiosus* epizootic in that fishery. Obviously other potential sources of *B. exitiosus* -free oysters needed to be investigated. The most promising, readily available source was a population of pre-recruit sized oysters cultured by a commercial supplier in the Marlborough Sounds. Screening of 60, two year old oysters from this population for *B. exitiosus* by histology and heart smears found no positives. These data indicated that, if *B. exitiosus* was present in the pre-recruit oysters, statistically there was 95% confidence it was present at a prevalence of less than 5%, assuming the diagnostic methods used were 100% effective. Pre-recruit sized oysters from this population were subsequently used in the second experiment, despite the possibility that the smaller oyster size might have been a confounding factor, given that older oysters tend to be more susceptible to bonamiosis (van Banning 1990, Culloty and Mulcahy 1996).

Pre recruit oysters: The pre-recruit oysters proved susceptible to bonamiosis, hence the results from the second experiment were an improvement over those of the first. Nevertheless, control oysters were still found to be infected with *B. exitiosus* after 10 weeks. These data suggest that either the pre-recruit oysters had pre patent *B. exitiosus* infections at the start of the experiments, or else the oysters became infected with *B. exitiosus* during the experiments via the unfiltered water supply at Greta Point. Other researchers have maintained experimental oysters in filtered seawater in recirculation systems to prevent extraneous sources of infection (Hervio et al. 1995). In the present study however, the water used was unfiltered and sourced from Wellington Harbour, an area where *B. exitiosus* is enzootic. Control pre-recruit oysters began dying from moderate *B. exitiosus* infections after 10 weeks, and heavy *B. exitiosus* infections in control oysters were recorded after 15 weeks (Figure 10). These data approximately correspond to the 3-4 month lag period described when naïve European flat oysters are exposed to *B. ostreae* (see Tige and Grizel 1984, Montes 1991), suggesting that in the present study the control oysters appeared most likely to have been infected by *B. exitiosus* via the unfiltered water supply during the course of the experiment.

The relatively high prevalence of bonamiosis and the associated high mortality rates in control oysters, compared to the oysters injected with 10^2 and 10^4 *B. exitiosus*, may be due to increased stress associated with the 200 and 300 µl volumes of filtered seawater administered to some of the control oysters. Pre-recruit oysters injected with up to 10^5 *B. exitiosus* were injected with only 100µl of *B. exitiosus* inoculum (as per Hervio et al. 1995) prepared by dilution of the typical yield of 3 - 4 x 10^6 *B. exitiosus* in 1 ml of purification product. However, for the 10^6 dose the 1 ml yield from purifications could provide enough *B. exitiosus* for only 3 or 4 oysters, hence the inoculum volume was adjusted to 200-300 µl / oyster. To control for the increased inoculum volume used in the 10^6 treatment, 5 control oysters were inoculated with 200µl and 5 with 300µl of filtered seawater. It is possible that the stress associated with the increased inoculum volume in these oysters predisposed them to infection with *B. exitiosus* above that of the control oysters injected with only 100µl of filtered seawater, and also above that of the treatment oysters injected with 100 µl of inoculum containing 10^2 and 10^4 *B. exitiosus*.

If it is assumed that the water supply was the source of the bonamiosis in the control oysters, and that this effect was uniform across all treatments, the results from the second experiment with the pre-recruit oysters could still provide some useful data.

Firstly, mortality above that of control oysters was recorded for dose rates above 10^4 *B. exitiosus* / oyster, which may suggest that the background dose each oyster was exposed to via the unfiltered water was in the order of 10^3 - 10^4 *B. exitiosus* / oyster. Experimental doses in the 10^5 and 10^6 treatments may, therefore, be cautiously assessed on their merits as the variation in dose due to the unfiltered water supply probably caused errors in the order of 10% and 1% of the experimental dose, respectively, which fall within acceptable error limits for the purposes of this preliminary study. This indicates that the calculations for the 18 week 50% lethal dose of *B. exitiosus* (111,200 parasites ± 10%), which was based mainly on data from the 10^5 and 10^6 treatments using the Reed and Muench (1938) method, may be a reasonable representation of the 18 week LD 50 for oysters in the pre-recruit size range. For comparison Hervio et al. (1995) found the 50% infectious dose for *B. ostreae* to be 80,000 parasites for 3 year old European flat oysters.

When the results of experiments by Hervio et al. (1995), using a 100% infective dose (2.2 x 10^5 *B. ostreae* / 3 year old oyster) at 18°C are compared to a similar dose rate from the present study (1 x 10^5 *B. exitiosus* /2 year old pre-recruit oyster, 15°C), the cumulative mortalities after 4 months are similar (Figure 18). The higher dose rate, higher temperature and older oysters used in the experiments with *B. ostreae* corresponded to a similar mortality rate after 16 weeks (42%),

compared to 45% for 18 weeks using *B. exitiosus*. These data suggest that the 16-18 week LD 50 rates for both parasites are probably similar, and are in the order of 1.1 - 3 x 10^5 parasites /oyster for 2 - 3 year old oysters.

The second aspect for which the data from the experiment with pre-recruit oysters are useful is for determination of the onset of mortality after exposure to *B. exitiosus*. This may still be estimated because the time of first exposure to significant levels of *B. exitiosus* can be assumed to be the beginning of the experiment. From these data this would indicate that the onset of mortalities due to bonamiosis might begin as early as 2.5 months after exposure to 10^4 *B. exitiosus* / oyster, and perhaps as early as 1.5 months after exposure to 10^5 *B. exitiosus*. Oysters injected with 10^6 *B. exitiosus*, (or approximately 9 times the estimated 4 month 50% lethal dose), were recorded dying with heavy *B. exitiosus* infections within the first week post-exposure.

The major drawbacks in this experimental design included the inability to obtain a reliable supply of *B. exitiosus*-free oysters (at least for recruit sized oysters from Foveaux strait), the difficulty purifying sufficient *B. exitiosus* to inoculate all the experimental oysters at higher dose rates (e.g. the 10^6 *B. exitiosus*/ oyster treatment), the lack of filtration of incoming water (which exposed experimental oysters to *B. exitiosus* via the water supply), and the inability to replicate each treatment (due to logistical difficulties associated with insufficient tank space). The original experimental design for this research objective called for a repeat of this experiment at a lower water temperature (8°C) to examine how temperature influences the course of *B. exitiosus* infection. Drawing from the experience gained during the course of the experiments at 15°C, we recommend that if the experiments at 8°C are undertaken (or if the experiments at 15°C are to be repeated), a revised experimental protocol is used so that:

1. The experiments are conducted using *B. exitiosus*-free oysters, either pre-recruit cultured oysters from the Marlborough Sounds (after screening for *B. exitiosus* by statistical testing at the 5% disease incidence level), or if recruit sized oysters are required, only after a supply of *B. exitiosus*-free recruit sized oysters is located by statistical testing at the 5% disease incidence level.
2. A reliable supply of heavily infected oysters is sourced to allow recovery of higher parasite yields from purifications to allow the full number of oysters to be inoculated in the 10^6/oyster dose rate.
3. That the experiments are conducted in a filtered water supply (which would then entail production of algae to feed oysters for the 4 month duration of the experiment), or in areas where *B. exitiosus* is not enzootic, to exclude infective stages from the water supply.
4. That sufficient tanks are available to allow replication of the treatments at least twice.

Objective 8: Summary

The estimation of the 18 week 50% lethal dose of *B. exitiosus* in pre-recruit sized oysters was around 1.12 x 10^5 parasites (± 10%). The onset of mortalities of pre-recruit sized oysters due to bonamiosis appears to be between 3 and 4 months for naïve pre-recruit oysters exposed to environmental levels of *B. exitiosus* via the water, but appears to begin as early as 2.5 months after exposure to 10^4 *B. exitiosus* / oyster by injection, as early as 1.5 months after exposure to 10^5 *B. exitiosus* by injection, or as little as 1 week after exposure to 10^6 *B. exitiosus* by injection.

Objective 8: Recommendations:

If further experiments are undertaken at 8°C (or if the experiments at 15°C are to be repeated), a revised experimental protocol should be used so that:

1. **The experiments are conducted using *B. exitiosus*-free oysters, either pre-recruit cultured oysters from the Marlborough Sounds (after screening for *B. exitiosus* by statistical testing at the 5% disease incidence level), or if recruit sized oysters are required, only after a supply of *B. exitiosus*-free recruit sized oysters is located by statistical testing at the 5% disease incidence level.**
2. **A reliable supply of heavily infected oysters is sourced to allow recovery of higher parasite yields from purifications to allow the full number of oysters to be inoculated in the 10^6/oyster dose rate.**

3. **That the experiments are conducted in a filtered water supply (which would entail feeding oysters algae for the 4 month duration of the experiment), or in areas where *B. exitiosus* is not enzootic, to exclude infective stages from the water supply.**

4. **That sufficient tanks are available to allow replication of the treatments at least twice.**

Table 11. Size (recruit/ pre–recruit), *Bonamia exitiosus* concentration and volume of inoculum injected into dredge oysters used for Objective 8 experiments.

Oyster size, inoculation vol	Mean oyster size (mm)	Tank 1 (10^2 *Bonamia*)	Tank 2 (10^4 *Bonamia*)	Tank 3 (10^5 *Bonamia*)	Tank 4 (10^6 *Bonamia*)	Tank 5 (FSW only)
Number of recruited oysters	83.3 x 65.1	20 (200µl)	20 (200µl)	20 (200µl)	–	20 (200µl)
Number of pre-recruit oysters	54.4 x 43.8	10 (100µl)	10 (100µl)	20 (100µl)	15 (200 – 300µl)	20 (100 - 300 µl)

Table 12. Calculation of the 50% lethal dose of *B. exitiosus* for pre-recruit oysters (see Reed and Muench (1938) for details) during an 18 week observation period at 15°C.

No. *B. exitiosus* injected	no. oysters surviving	no. oysters dead	Calculated no. oysters alive	Calculated no. of oysters dead	% of dead oysters
10^1	0	0	30	0	0
10^2	9	1	30	1	3.2
10^3	0	0	21	1	4.5
10^4	7	3	21	4	16
10^5	11	9	14	13	48.1
10^6	3	12	3	25	89.3
Proportional distance = (50 - 48.1)/(89.3 - 48.1) = 0.046. 50% end point = $10^{(\text{lower dilution + proportional distance})} = 10^{(5 + 0.046)} = 10^{5.046}$ 4 month LD50 = 111,200 *B. exitiosus*.					

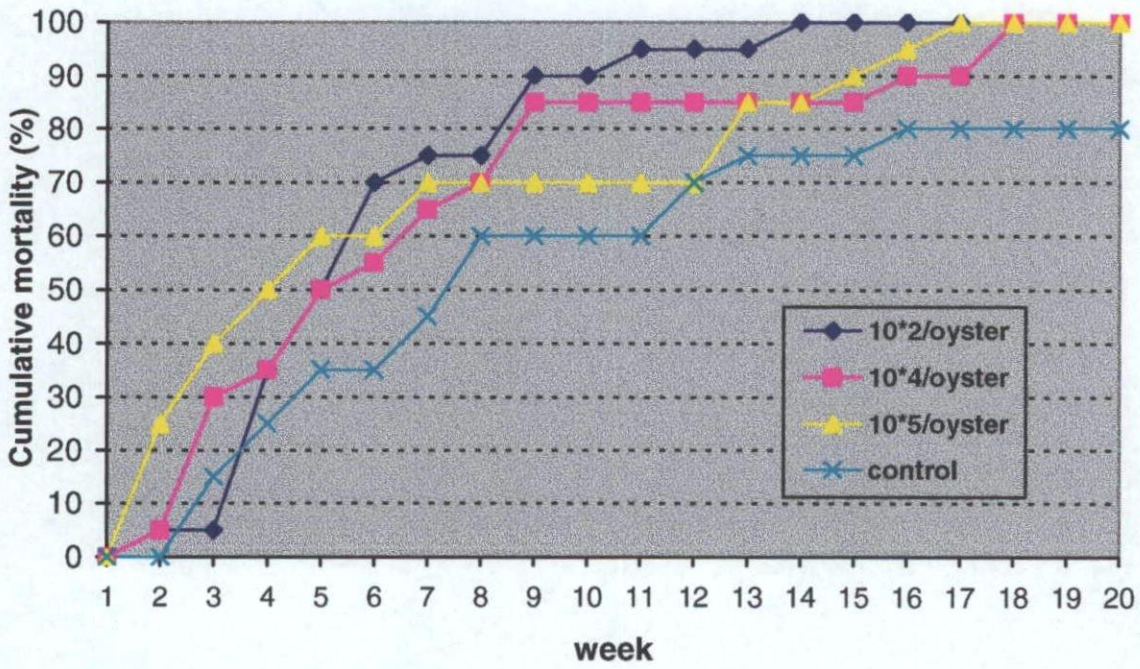

Figure 3. Cumulative mortality (%) during 20 weeks observation at 15°C of recruited oysters injected with either 10^2 (dark blue diamonds), 10^4 (pink squares) or 10^5 (yellow triangles) purified viable *B. exitiosus*, and control oysters (light blue crosses) injected with 0.22 µm filtered seawater.

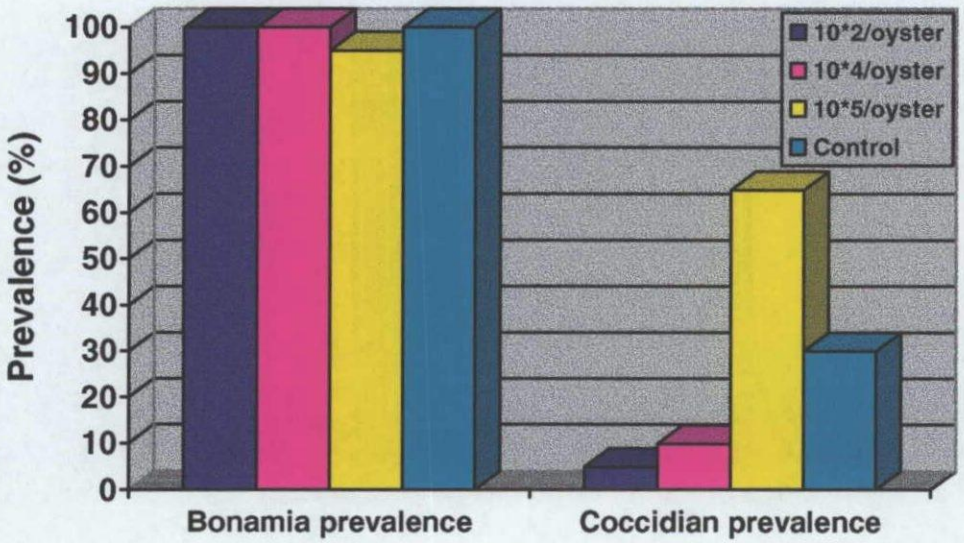

Figure 4. Prevalence of *B. exitiosus* and an unnamed coccidian parasite in recruited oysters injected with either 10^2 (dark blue), 10^4 (pink) or 10^5 (yellow) purified viable *B. exitiosus*, and control oysters (light blue) injected with 0.22 µm filtered seawater.

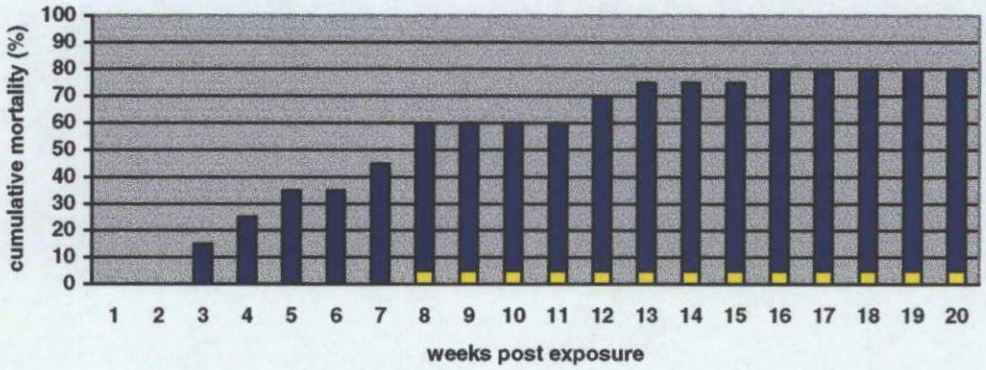

Figure 5. Cumulative mortalities for control recruited oysters injected with filtered seawater. Yellow = light *B. exitiosus* infection (grades 1-2), Dark blue = heavy *B. exitiosus* infection (grades 4-5).

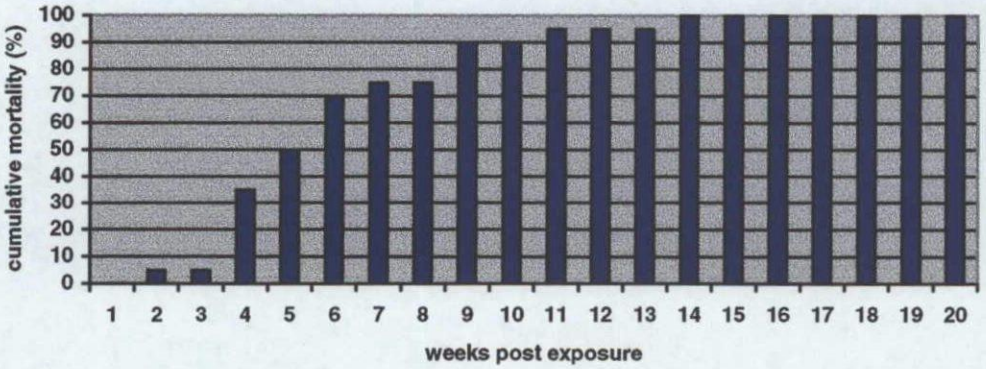

Figure 6. Cumulative mortalities for recruited oysters injected with 10^2 *B. exitiosus*. Dark blue = heavy *B. exitiosus* infection (grades 4-5).

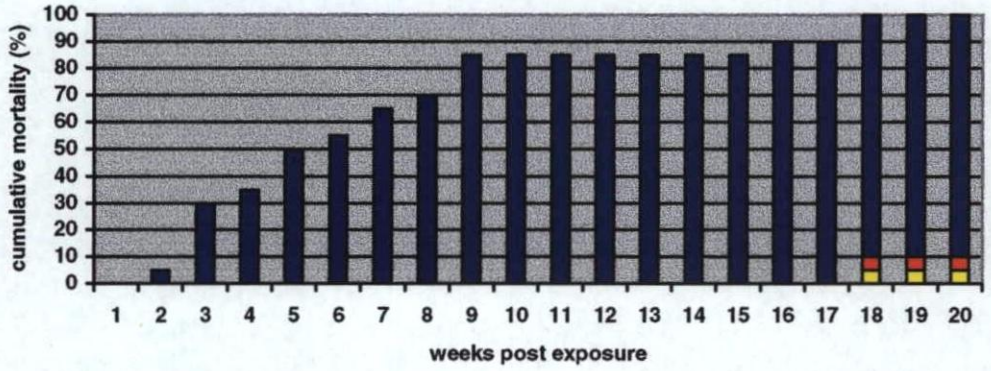

Figure 7. Cumulative mortalities for recruited oysters injected with 10^4 *B. exitiosus*. Yellow = light *B. exitiosus* infection (grades 1-2), Red = moderate *B. exitiosus* infection (grade 3), Dark blue = heavy *B. exitiosus* infection (grades 4-5).

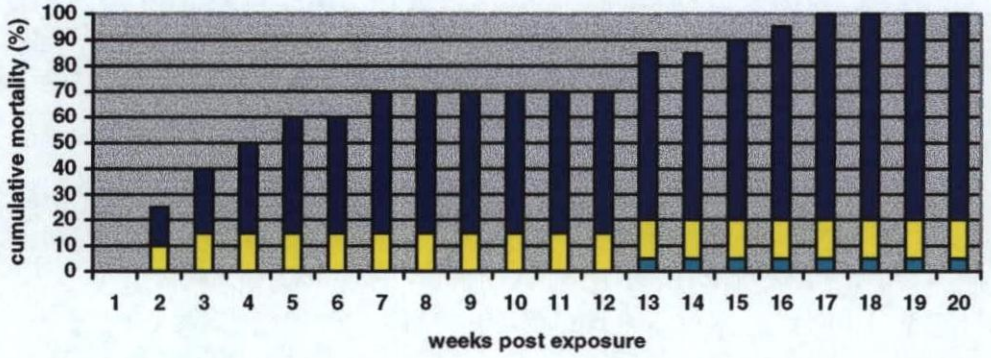

Figure 8. Cumulative mortalities for recruited oysters injected with 10^5 *B. exitiosus*. Light blue = not infected, Yellow = light *B. exitiosus* infection (grades 1-2), Dark blue = heavy *B. exitiosus* infection (grades 4-5).

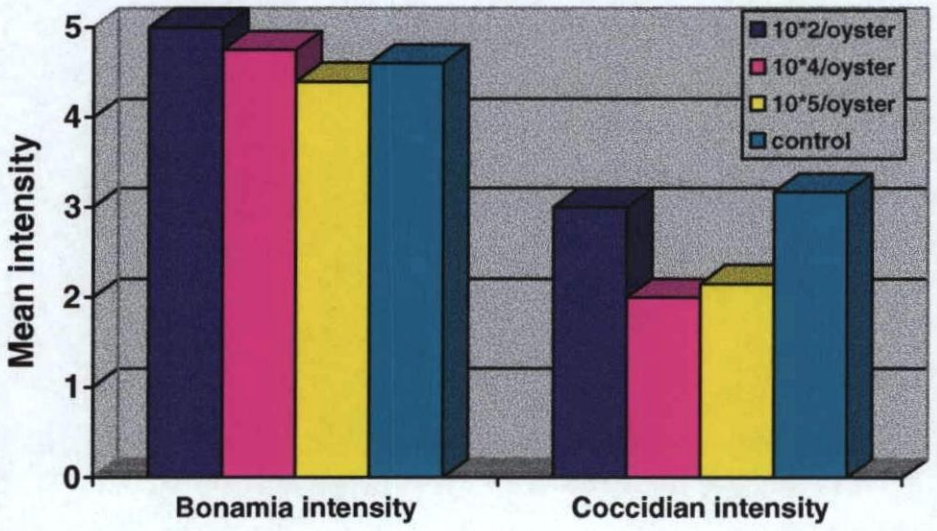

Figure 9. Mean intensity of infections of *B. exitiosus* and an unnamed coccidian parasite in recruited oysters injected with 10^2 (dark blue), 10^4 (pink) or 10^5 (yellow) purified viable *B. exitiosus*, and control oysters (light blue) injected with 0.22 µm filtered seawater.

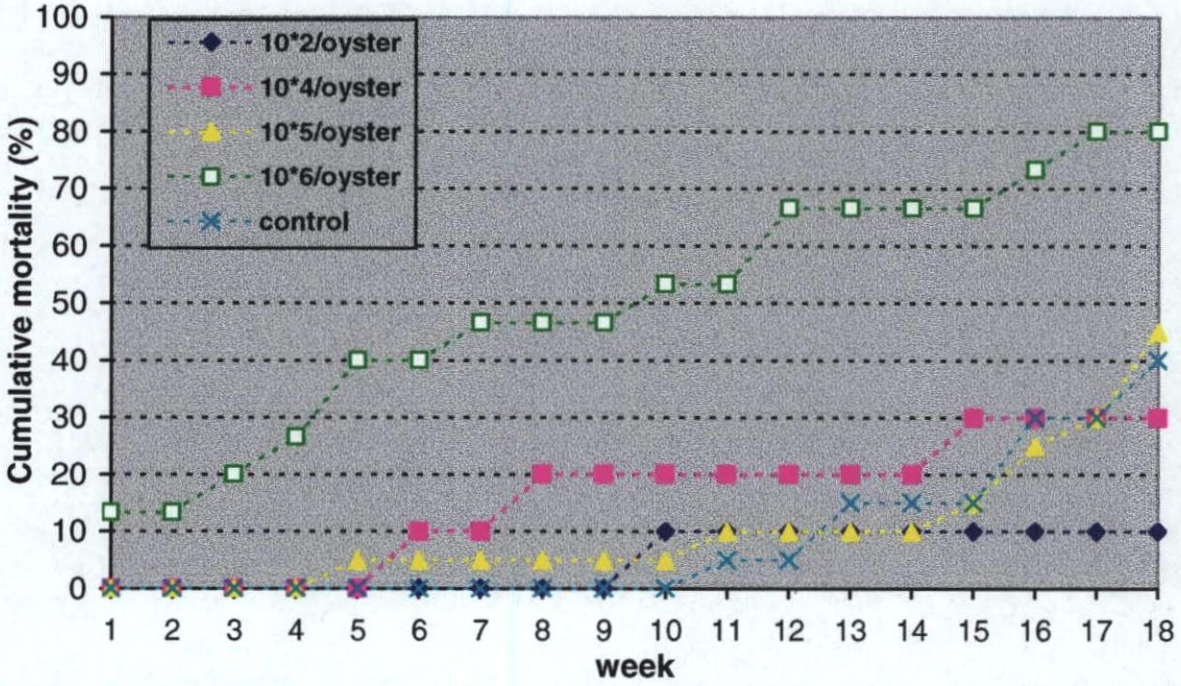

Figure 10. Cumulative mortality (%) during 18 weeks observation at 15°C of pre–recruit oysters injected with either 10^2 (dark blue diamonds), 10^4 (pink squares), 10^5 (yellow triangles) or 10^6 (hollow green squares) purified viable *B. exitiosus*, and control oysters (light blue crosses) injected with 0.22 µm filtered seawater.

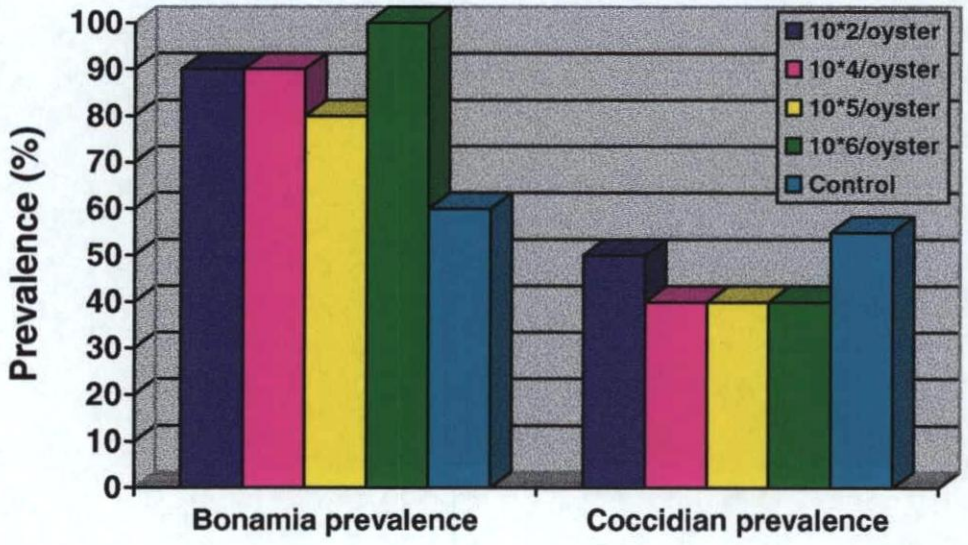

Figure 11. Prevalence of *B. exitiosus* and an unnamed coccidian parasite in pre-recruit oysters injected with either 10^2 (dark blue), 10^4 (pink), 10^5 (yellow) or 10^6 (green) purified viable *B. exitiosus*, and control oysters (light blue) injected with 0.22 µm filtered seawater.

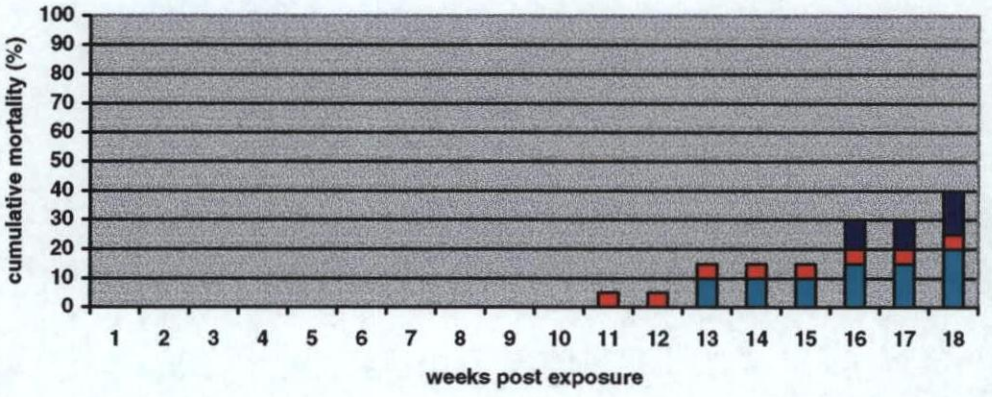

Figure 12. Cumulative mortalities for control pre-recruit oysters injected with filtered seawater. Light blue = not infected, Red = moderate *B. exitiosus* infection (grade 3), Dark blue = heavy *B. exitiosus* infection (grades 4-5).

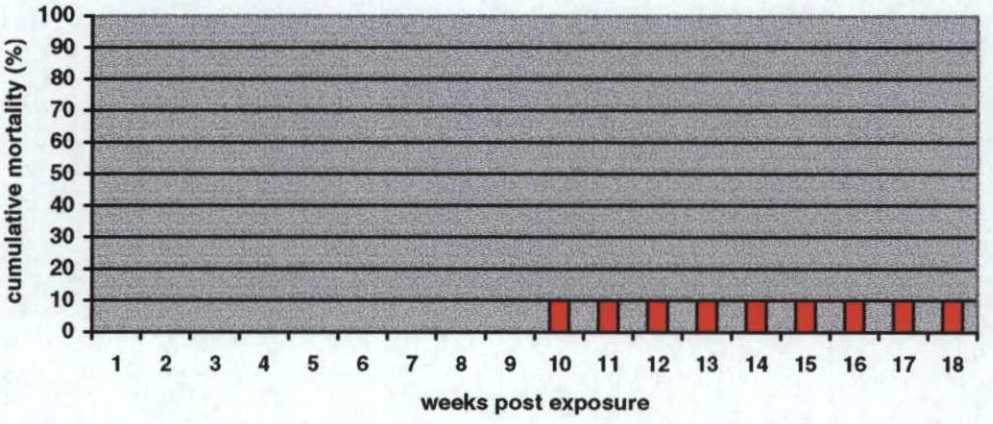

Figure 13. Cumulative mortalities for pre-recruit oysters injected with 10^2 *B. exitiosus*. Red = moderate *B. exitiosus* infection (grade 3).

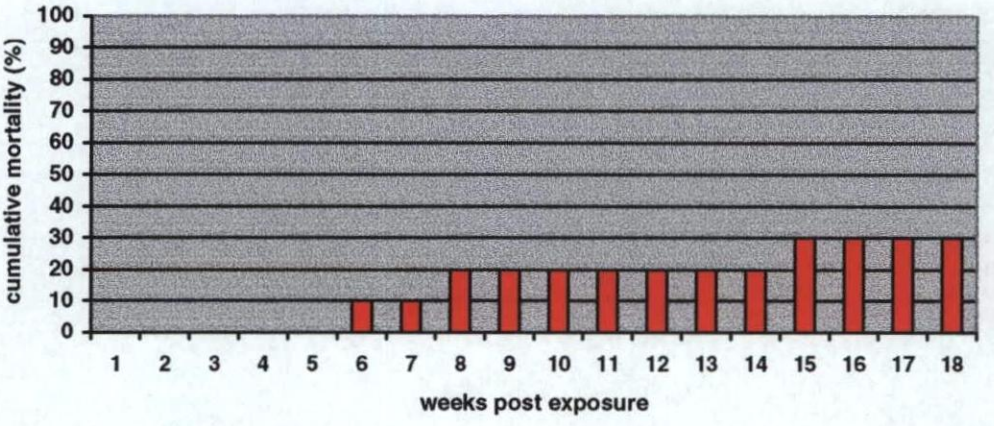

Figure 14. Cumulative mortalities for pre-recruit oysters injected with 10^4 *B. exitiosus*. Red = moderate *B. exitiosus* infection (grade 3).

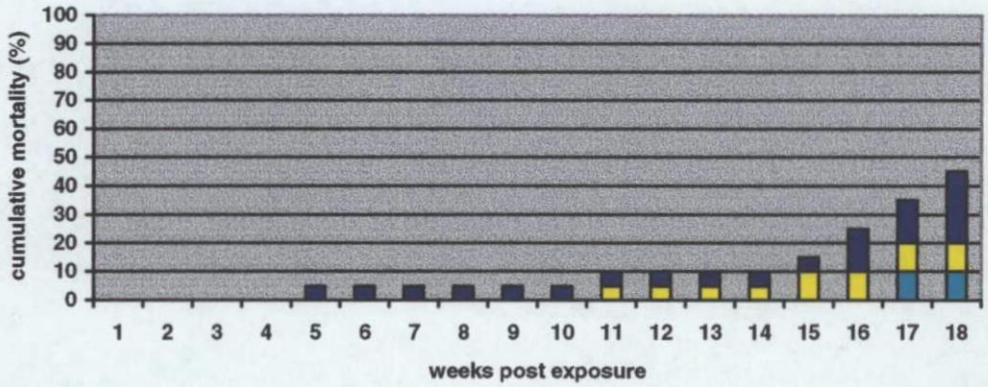

Figure 15. Cumulative mortalities for pre-recruit oysters injected with 10^5 *B. exitiosus*. Light blue = not infected, Yellow = light *B. exitiosus* infection (grades 1-2), Dark blue = heavy *B. exitiosus* infection (grades 4-5).

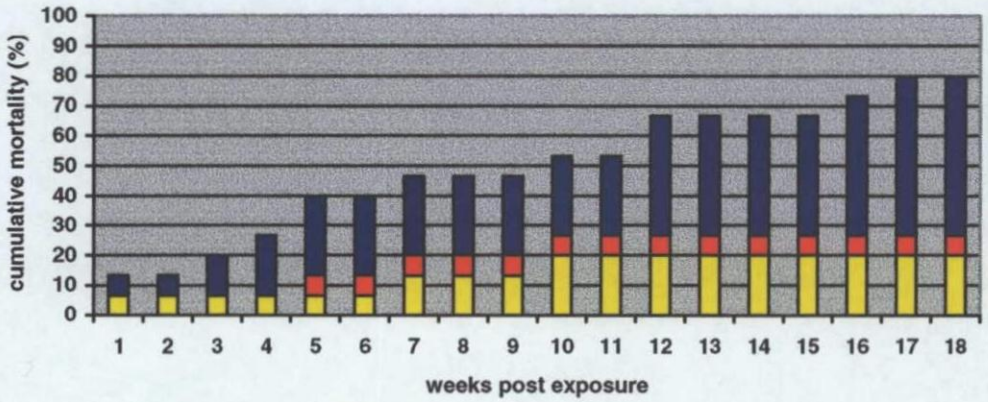

Figure 16. Cumulative mortalities for pre-recruit oysters injected with 10^6 *B. exitiosus*. Yellow = light *B. exitiosus* infection (grades 1-2), Red = moderate *B. exitiosus* infection (grade 3), Dark blue = heavy *B. exitiosus* infection (grades 4-5).

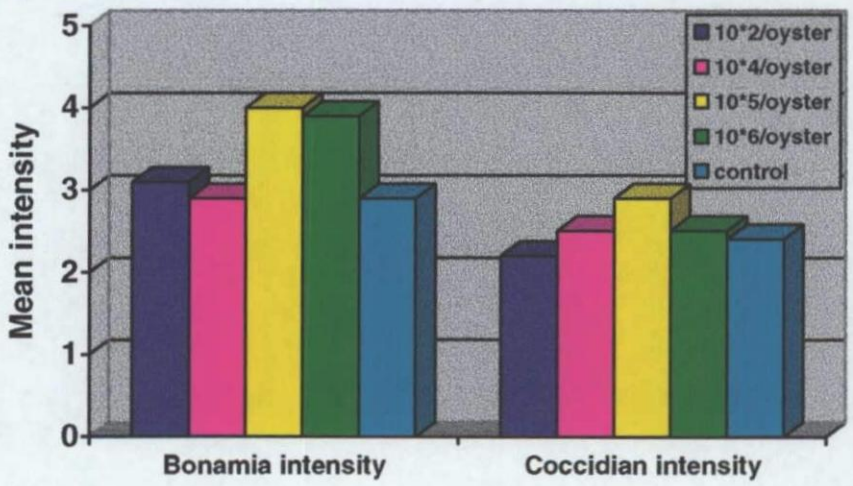

Figure 17. Mean intensity of infections of *B. exitiosus* and an unnamed coccidian parasite in pre-recruit oysters injected with either 10^2 (dark blue), 10^4 (pink), 10^5 (yellow) or 10^6 (green) purified viable *B. exitiosus*, and control oysters (light blue) injected with 0.22 µm filtered seawater.

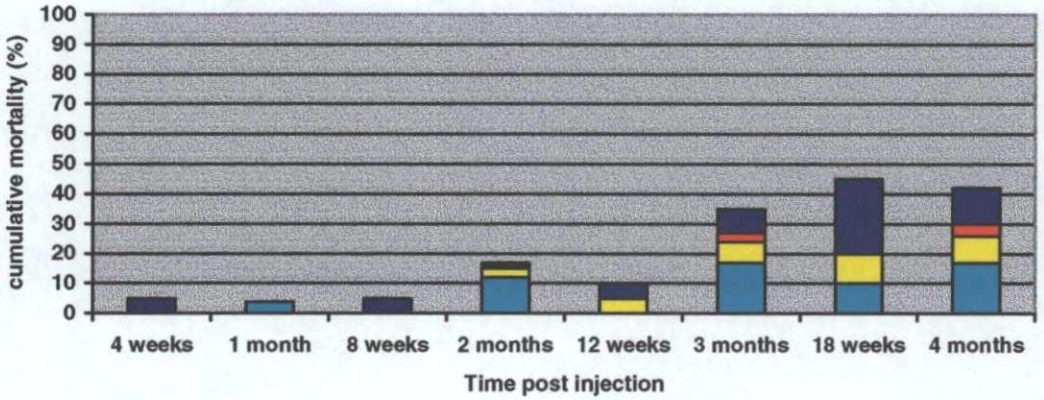

Figure 18. Comparison of cumulative mortality rates of flat oysters injected with 1 x 10^5 *B. exitiosus* (present study, host *Ostrea chilensis*, 15°C, time in weeks) against European flat oysters given a 100% infectious dose (2.2 x 10^5) of *Bonamia ostreae* (data from Hervio *et al.* 1995, Figure 3, host *Ostrea edulis*, 18°C, time in months). Light blue = not infected, Yellow = light *Bonamia* infection (grades 1-2), Red = moderate *Bonamia* infection (grade 3), Dark blue = heavy *Bonamia* infection (grades 4-5).

OBJECTIVE 9:

To determine how long *Bonamia exitiosus* can survive outside the host.

Introduction:

There are two facets to survival of *B. exitiosus* outside the host; survival in the water column during transmission between hosts, and survival on boats and fishing gear when the parasite will be exposed to partial desiccation and ultraviolet light. *Bonamia* has no known spore stage resistant to desiccation, and the stage that exists outside the host is a small (~2 μm) protoplasmic stage. It therefore appears unlikely that it can survive for long out of water, particularly in only semi–moist conditions, or exposed to UV light. Survival of *B. exitiosus* in seawater, however, is unknown, and these data are important for development of the infection model. The host, *Ostrea chilensis,* is known to be able to survive a wide range of temperatures (6–23°C) and salinities (8–33‰) (Buroker et al. 1983).

Method:

Viable purified *B. exitiosus* were held in 0.2 μm filtered seawater of various salinities (5, 15, 25 and 36‰ salinity), for different periods of time (1, 2, 4, 6, 12, 24, 48 and 72 hours) at a range of temperatures (4, 10 and 18°C), in sterile plastic 48 well trays (Nunclon™). Before being placed in the wells, a sub sample of purified *B. exitiosus* was counted with a hemocytometer and their viability was assessed using trypan blue exclusion. The *B. exitiosus* which took up trypan blue dye were considered unviable, while those that did not were considered viable. The concentration of viable *B. exitiosus* was adjusted to 10^5 *B. exitiosus* / ml with 0.2 μm filtered seawater and 1 ml of this solution was placed in each well as shown in Table 13. Trays containing replicate rows of wells containing 10^5 *B. exitiosus* at different salinities were incubated at their respective temperatures. At each time period the trays were placed over an inverted microscope and the viability of *B. exitiosus* in one column of wells was assessed by placing 10 μl of trypan blue into the appropriate wells and counting the proportion of viable and unviable cells in the first 100 *B. exitiosus* observed. Each variable was replicated twice to obtain values for mean survival of purified *B. exitiosus* for each temperature/salinity/time combination.

Results:

Mean survival of *B. exitiosus* at 5‰ salinity after 1 hour was 74% at 4°C, 42% at 10°C and 25% at 18°C (Figures 19–21). Less than 5% survival was observed after 4 hours at 4°C and 10°C, and 6 hours at 18°C. Total mortality was recorded after 72 hours at both 10 and 18°C (Figures 20, 21).

Mean survival of *B. exitiosus* at 15‰ salinity after 1 hour reduced from 60% at 4°C, to 52% at 10°C and 17% at 18°C (Figures 19-21). Survival remained around 20% at 18°C until 48 hours, when it dropped to less than 1%. At 10°C survival ranged between 35 and 62% until 12 hours, when it dropped to around 20%, where it remained until the end of the experiment. At 4°C survival remained between 20 and 40% until 72 hours when it dropped to 6%.

Mean survival of *B. exitiosus* at 25‰ salinity remained between 80 and 90% from 1 to 24 hours at all three temperatures (Figures 19-21). At 48 hours, however, survival had dropped to around 60% at 10°C and 12% at 18°C. After 72 hours survival had dropped to 56 and 57% at 4 and 10°C, respectively, and was 0% at 18°C.

Mean survival of *B. exitiosus* at 36‰ salinity remained around 80 and 90% from 1 to 24 hours at all three temperatures (Figures 19-21). At 48 hours survival had dropped to 75% at 10°C and 60% at 18°C, but remained above 80% at 4°C. After 72 hours survival at 4°C and 10°C had dropped to 65 and 75%, respectively, while at 18°C survival was only 2%.

Discussion:

These results indicate that survival of *B. exitiosus* in seawater is highest at normal salinities (36‰), and was prolonged *in–vitro* by lower water temperatures. Survival remained relatively high at 25‰ salinity, but dropped markedly at 15‰ salinity and below. In full strength seawater (36‰ salinity), it appears that survival at 18°C begins to drop between 24 and 48 hours, with a mean 60% survival at 48 hours. The remaining half of the *B. exitiosus* died between 48 and 72 hours, suggesting that 50% survival time of waterborne *B. exitiosus* during the summer months is around 48 hours. At 4 and 10°C however, survival dropped only slightly between 24 and 48 hours, remaining above 65% after 72 hours. This suggests that over half of waterborne *B. exitiosus* can survive at least 4 days during the winter months.

Objective 9: Summary:

At summer water temperatures (18°C), approximately 50 % of *B. exitiosus* can survive 48 hours. At winter water temperatures (4–10°C), approximately 50% of *B. exitiosus* can survive at least 4 days. Survival of *B. exitiosus* drops markedly at and below 15‰ salinity.

Table 13. Layout of 48 well sterile Nunclon™ plates used to investigate the viability of *B. exitiosus* at different temperatures and salinities. Each well contained 1 ml of 0.2 µm filtered seawater containing 10^5 *B. exitiosus*. Each plate was incubated at either 4, 10 or 18°C.

Checked at 1 hour	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours	72 hours
5‰ salinity	⇒	⇒	⇒	⇒	⇒	⇒	⇒
15‰ salinity	⇒	⇒	⇒	⇒	⇒	⇒	⇒
25‰ salinity	⇒	⇒	⇒	⇒	⇒	⇒	⇒
36‰ salinity	⇒	⇒	⇒	⇒	⇒	⇒	⇒
Empty well	⇒	⇒	⇒	⇒	⇒	⇒	⇒

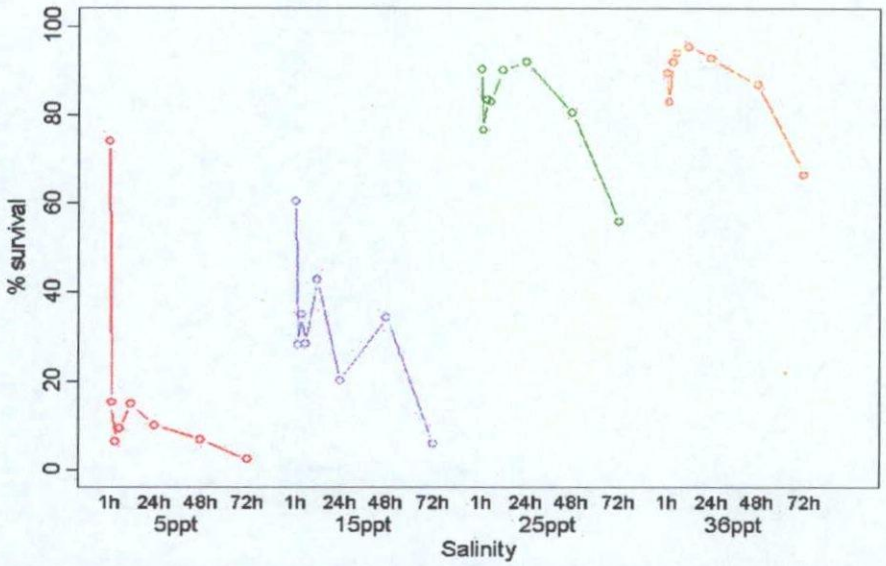

Figure 19. Mean survival (%) of purified *B. exitiosus* at different salinities in 4°C seawater.

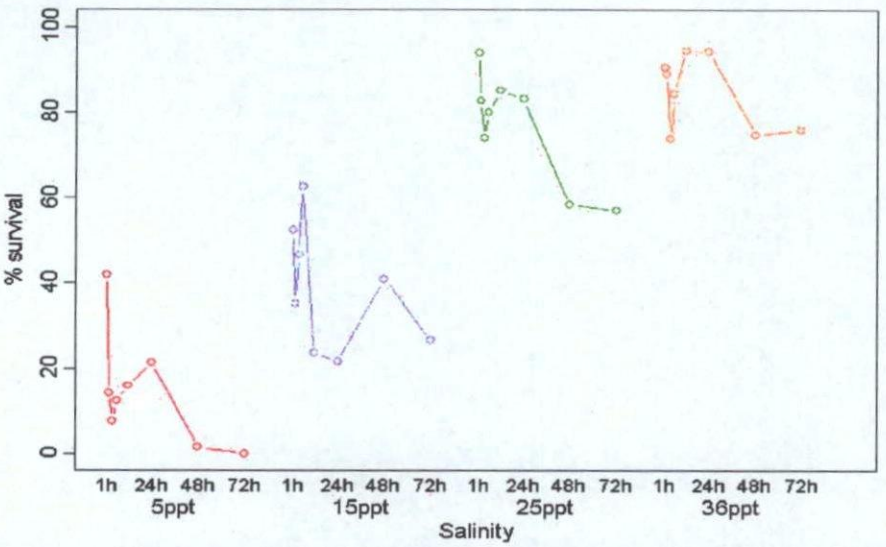

Figure 20. Mean survival (%) of purified *B. exitiosus* at different salinities in 10°C seawater.

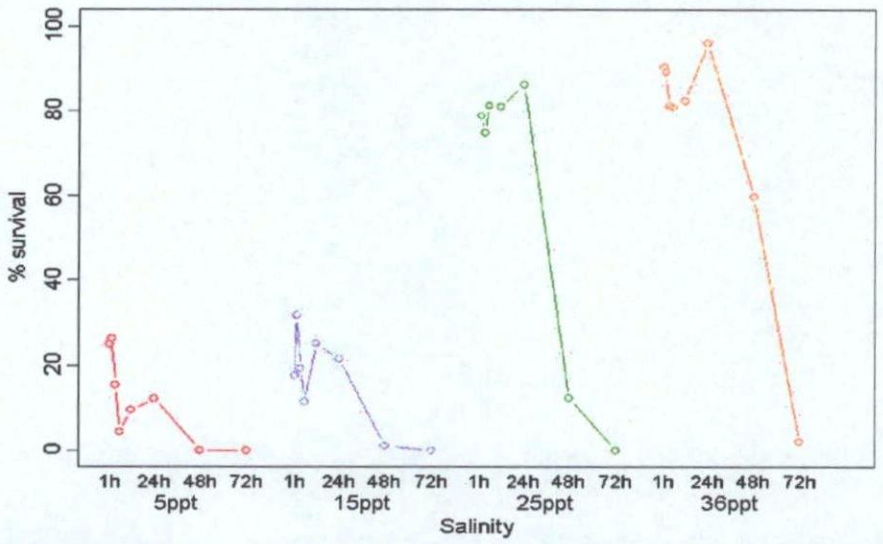

Figure 21. Mean survival (%) of purified *B. exitiosus* at different salinities in 18°C seawater.

OBJECTIVE 10:

To determine the distance over which *Bonamia exitiosus* can transmit between oysters.

Introduction:

To avoid the numerous logistical difficulties associated with large scale, expensive field experiments, oysters were exposed in small aquaria in the laboratory to different concentrations of viable purified *B. exitiosus* as a proxy for distance. The original aim of this methodology was to simulate the dilution of infective particles with distance from an oyster by exposing oysters to different parasite concentrations.

The major logistical problem which remained was location of suitable numbers of uninfected oysters. For this objective pre–recruit (< 58 mm wide) cultured oysters were obtained from a commercial supplier from the Marlborough Sounds after finding no evidence of *B. exitiosus* during sampling of 60 oysters from the facility by histology and heart smears. These data indicated that, if *B. exitiosus* was present at the facility, there was 95% confidence it was present at a prevalence of less than 5%, assuming the diagnostic methods used were 100% effective for detecting the presence of *B. exitiosus*.

Methods:

Groups of 30 live uninfected pre-recruit oysters (mean size 53.6 mm x 44.2 mm, n = 180) were each placed in small aquaria (40 x 20 x 20 cm) into which 3 x 10^2, 3 x 10^3, 3 x 10^4, 3 x 10^5 and 3 x 10^6 purified viable *B. exitiosus* were added to a volume of 20 litres of seawater at 15°C (Table 14). As there were 30 oysters in each aquaria, each oyster was exposed, on average, to 10^1, 10^2, 10^3, 10^4, and 10^5 viable *B. exitiosus*. The seawater in each aquarium was aerated for 24 h before the flow through water supply was reconnected, so the oysters were exposed to purified *B. exitiosus* for 24 hours. After this the oysters were reared for 18 weeks at 15°C. Another group of 30 oysters, held in 20 litres of non–circulating filtered seawater for 24 h at 15°C and then reconnected to the same flow through water supply for 18 weeks at 15°C, were used as controls. During this time the oysters were inspected daily and all gapers and dead oysters were removed and fixed for histology in 10% formalin in filtered seawater, and processed using standard histological techniques. Wax sections were stained with H&E and examined with the light microscope to determine the cause of death. After 18 weeks all surviving oysters were fixed as described above and examined by routine histology for the presence of *B. exitiosus*. Infections were graded using the same semiquantitative scoring methods used in Objective 8.

Results:

Mortality was highest in tank 5, where 40% of oysters exposed to 10^5 *B. exitiosus* died over the 18 week observation period. The next highest mortality rate (30%) was recorded for oysters in tanks 1,2, and 3 (10^1, 10^2, 10^3 *B. exitiosus* each, respectively), while 26.6% of the oysters exposed to 10^4 *B. exitiosus* died (Figure 22). Only 10% mortality was recorded in control oysters. The prevalence of *B. exitiosus* generally mirrored that of the intensity of exposure (Figure 23), with the highest prevalence of 73.3% being recorded in the oysters exposed to 10^5 *B. exitiosus*, except for tank 1 where the oysters exposed to 10^1 *B. exitiosus* had the second highest prevalence at 36.7% (Figure 23). Regression analysis showed that the increase in prevalence associated with exposure to higher numbers of *B. exitiosus* was not significant ((F = 0.1), mainly due to the anomalous result in tank 1.

When the dead oysters were examined for *B. exitiosus*, it was found that one of the oysters in tank 1which died during the first week post exposure had a heavy (grade 5) *B. exitiosus* infection (Figure 24). Another dead oyster moderately infected (grade 3) with *B. exitiosus* was also recorded in the first week of the experiment from tank 5 (10^5 *B. exitiosus* / oyster). Mortalities in tank 1 remained relatively low until weeks 8 - 10, after which the number of oysters dying with moderate to heavy infections of *B. exitiosus* increased (Figure 24). Oysters in most of the other treatment tanks showed similar trends with mortalities increasing around weeks 10- 12, and oysters with moderate to heavy *B. exitiosus* infections becoming more apparent between weeks 12 - 14 (Figures 25-28). Mortalities of control oysters in tank 6 were not associated with *B. exitiosus* infections that were detectable by histopathology (Figure 29), even though 3 of the surviving oysters in tank 6 had light to moderate *B. exitiosus* infections (Figures 23, 30).

The mean intensity of *B. exitiosus* in oysters from these experiments was only moderate, generally ranging between a score of 2 or 3, with the highest mean intensity of infection (4) being recorded in tank 3 for oysters exposed to 10^3 *B. exitiosus* (Figure 30). The prevalence and intensity of an unnamed coccidian parasite (Figures 23, 30) was similar to that of *B. exitiosus*, and it was difficult to determine whether the presence of the former influenced the mortalities observed.

Discussion:

Upon analysis of these results, it was clear that the experimental conditions used did not provide information which could be used to determine the transmission distance of *B. exitiosus*. Development of these data to a point where the transmission distance for *B. exitiosus* can be determined requires additional information, such as the number of *B. exitiosus* released when an oyster dies of bonamiosis, and knowledge of the patterns and mechanisms of dispersal of infective particles released from moribund and dead oysters. Nevertheless, the experiment still yielded some valuable information on the infectivity of waterborne *B. exitiosus*, results perhaps of more relevance to the situation in the wild than those provided by the objective 8 injection experiment. These results were therefore very useful to further define the lethal dose levels for pre-recruit oysters under conditions which approximate the natural conditions in the wild.

The fact that an oyster in tank 1 and another in tank 5 both died with moderate to heavy *B. exitiosus* infections during the first week post exposure strongly suggests that a low number of the experimental oysters had significant *B. exitiosus* infections prior to the start of the experiment. This was despite the negative result obtained during pre-experiment screening of 60 oysters from the same batch for *B. exitiosus* by histology and heart smears. These data suggest that *B. exitiosus* was present in the pre-recruit oysters, but at a prevalence of less than 5%.

Regression analysis of the data suggested there would have been a statistically significant increase in *B. exitiosus* prevalence with dose rate, except for an anomalous result in tank 1. In tank 1, it appeared the death of one heavily infected oyster during the early stages of the experiment precipitated an outbreak of bonamiosis in the remaining oysters in that tank. The mortality rate in tank 1 appeared to increase markedly at around 8 to 10 weeks post exposure, a time period similar to that found in the objective 8 experiment for pre-recruit oysters exposed to 10^4 *B. exitiosus* by injection. The final mortality level and prevalence of *B. exitiosus* for tank 1 oysters was also similar to those of oysters in tanks 3 and 4, which were exposed to 10^3 and 10^4 *B. exitiosus* respectively, via the water. These results suggest the oysters in tank 1 were exposed to additional *B. exitiosus*, perhaps in the order of an additional 10^2 to 10^3 *B. exitiosus* each, due to their close proximity to an oyster which died from pre-existing bonamiosis. Oysters in Tank 5 may also have been exposed to additional *B. exitiosus* from a previously infected oyster which died early in the experiment, however this did not appear to have a marked effect on the results,

probably because the dead oyster was a relatively minor source of *B. exitiosus* compared to the experimental dose in this treatment (10^5 *B. exitiosus* /oyster). These data suggest that future experiments which aim to infect oysters with *B. exitiosus* should be done under conditions where the deaths of individual oysters due to bonamiosis do not influence the dose rate experienced by the surviving oysters. One method of ensuring this would be to conduct experiments with oysters held in individual containers with their own individual filtered water supply.

The control oysters in these experiments appeared to be affected little, if at all, by the presence of *B. exitiosus* in the unfiltered water supply, in contrast to the pre-recruit control oysters used in the objective 8 experiments. Only 10% of control oysters had light to moderate *B. exitiosus* infections at the end of the experiment, but none of the 10% of control oysters which died had detectable *B. exitiosus* infections. This may be due to the fact that the control oysters in these experiments were not exposed to invasive procedures. Perhaps the process of drilling and injecting control oysters with large volumes (100-300µl) of filtered sea water in objective 8 experiments caused additional stress and thus predisposed the pre-recruit control oysters in that experiment to bonamiosis.

In tank 3 (10^3 *B. exitiosus* /oyster) there was a gradual increase in mortalities of oysters with light *B. exitiosus* infections from week 8, while heavily infected oysters being first noted in week 13. In tanks 4 and 5 (10^4 and 10^5 *B. exitiosus* /oyster, respectively), there were marked increases in mortality rates of oysters with *B. exitiosus* infections between weeks 10 to 12. These results approximate the beginning of the 3 month lag period described when naïve European flat oysters are exposed to *B. ostreae* (see Tige and Grizel 1984, Montes 1991), while Hervio et al. (1995) found that most European flat oysters (*Ostrea edulis*) experimentally infected with an 100% infective dose of *Bonamia ostreae* died of bonamiosis no earlier than 2 months after infection, and that most mortalities occurred after 3 or 4 months at 18°C. It seems likely, therefore, that exposure to 10^3 to 10^5 *B. exitiosus* via the water is sufficient to cause mortality in 25 to 40% of pre recruit oysters within 18 weeks. These results suggest that the 18 week LD 50 for oysters exposed to *B. exitiosus* via the water is probably only slightly higher than that found in Objective 8 for oysters injected with *B. exitiosus*, i.e. around 2 - 3 x 10^5 *B. exitiosus*/ oyster.

Objective 10: Summary

Development of these data to a point where the transmission distance for *B. exitiosus* can be determined requires additional information on the number of *B. exitiosus* released when an oyster dies of bonamiosis, and knowledge of the patterns and mechanisms of dispersal of infective particles released from moribund and dead oysters.

The experimental results suggest that exposure to 10^3 to 10^5 *B. exitiosus* via the water is likely to cause mortalities of 25 to 40% of pre recruit oysters within 18 weeks. These results suggest that the 18 week LD 50 for oysters exposed to *B. exitiosus* via the water is only slightly higher than that found in Objective 8 for oysters injected with *B. exitiosus*, i.e. around 2 - 3 x 10^5 *B. exitiosus*/ oyster.

The control oysters in these experiments appeared to be affected little, if at all, by the presence of *B. exitiosus* in the unfiltered water supply, possibly due to the fact they were not exposed to additional stress associated with invasive procedures.

Objective 10: Recommendations

Future experiments which aim to infect oysters with *B. exitiosus* should be done under conditions where the deaths of individual oysters due to bonamiosis do not influence the dose rate experienced by the surviving oysters.

Table 14. Number of pre–recruit oysters exposed to various concentrations of viable *B. exitiosus* in 20 litres of seawater at 15°C for Objective 10 experiments.

Tank 1 (10^1 *Bonamia* per oyster)	Tank 2 (10^2 *Bonamia* per oyster)	Tank 3 (10^3 *Bonamia* per oyster)	Tank 4 (10^4 *Bonamia* per oyster)	Tank 5 (10^5 *Bonamia* per oyster)	Tank 6 (seawater only, control)
30 oysters, 3×10^2 purified *Bonamia*	30 oysters, 3×10^3 purified *Bonamia*	30 oysters, 3×10^4 purified *Bonamia*	30 oysters, 3×10^5 purified *Bonamia*	30 oysters, 3×10^6 purified *Bonamia*	30 oysters filtered sea water

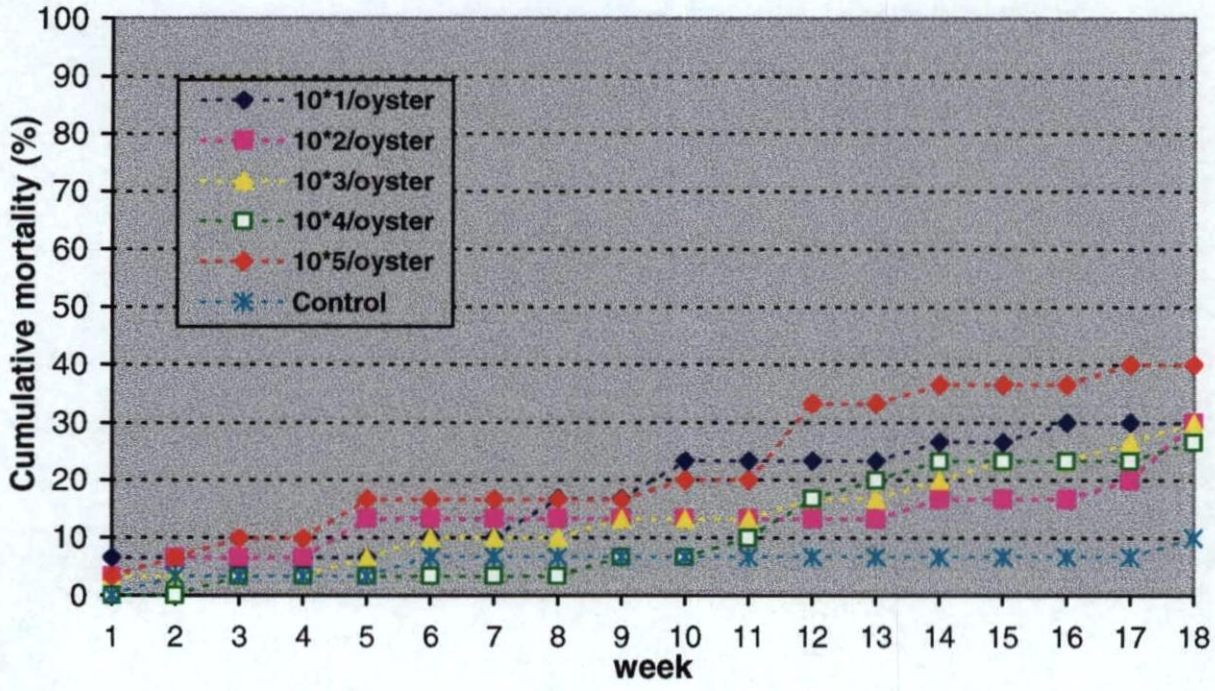

Figure 22. Cumulative mortality (%) during 18 weeks observation at 15°C of pre–recruit oysters exposed to either 10^1 (dark blue diamonds), 10^2 (pink squares), 10^3 (yellow triangles), 10^4 (green squares) or 10^5 (red diamonds) purified viable *B. exitiosus* in static aquaria for 24 hours, and control oysters (light blue stars) exposed to clean seawater.

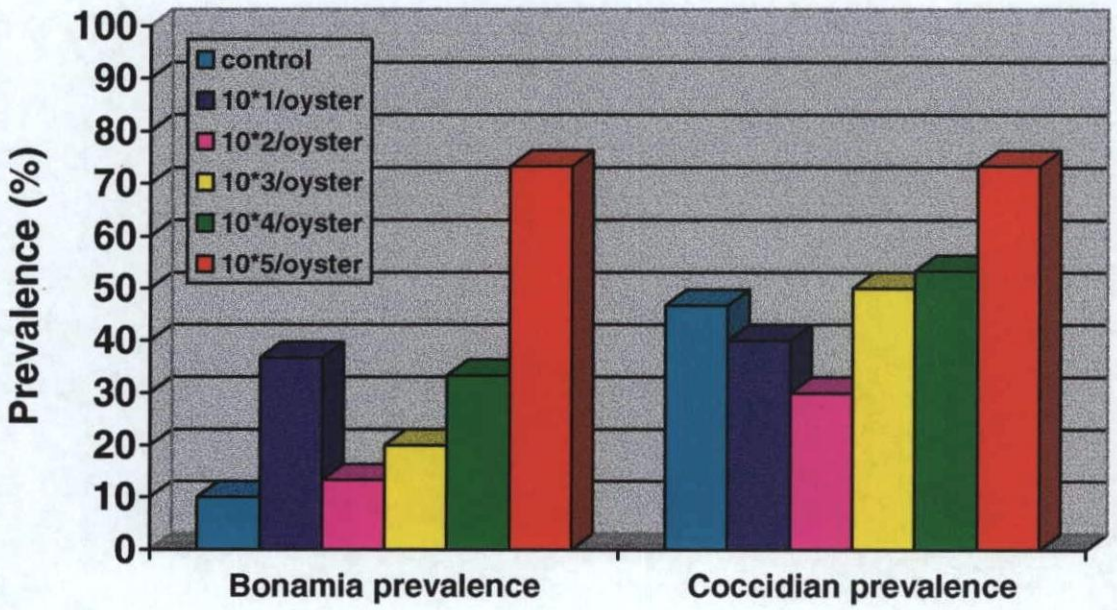

Figure 23. Prevalence of *B. exitiosus* and an unnamed coccidian parasite in pre-recruit oysters exposed via the water to either 10^1 (dark blue), 10^2 (pink), 10^3 (yellow), 10^4 (green), or 10^5 (red) purified viable *B. exitiosus*, and control oysters (light blue) exposed to clean seawater.

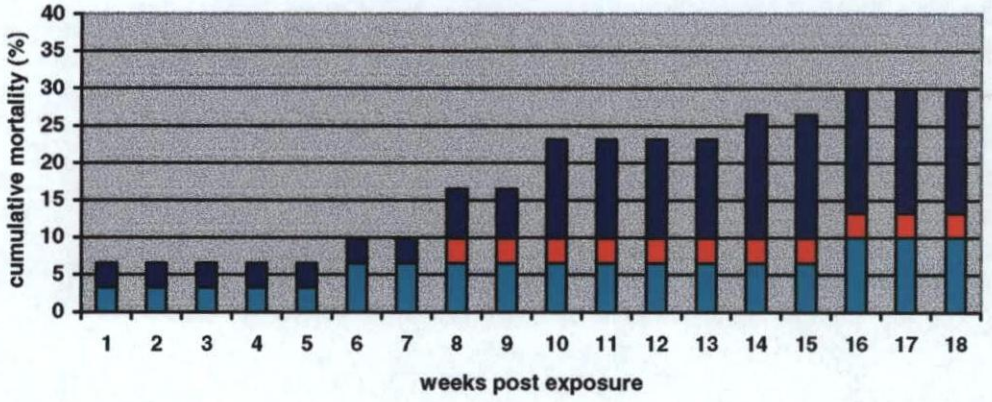

Figure 24. Cumulative mortalities for pre-recruit oysters in tank 1 exposed to 10^1 *B. exitiosus* via the water. Light blue = not infected, Red = moderate *B. exitiosus* infection (grade 3), Dark blue = heavy *B. exitiosus* infection (grades 4-5).

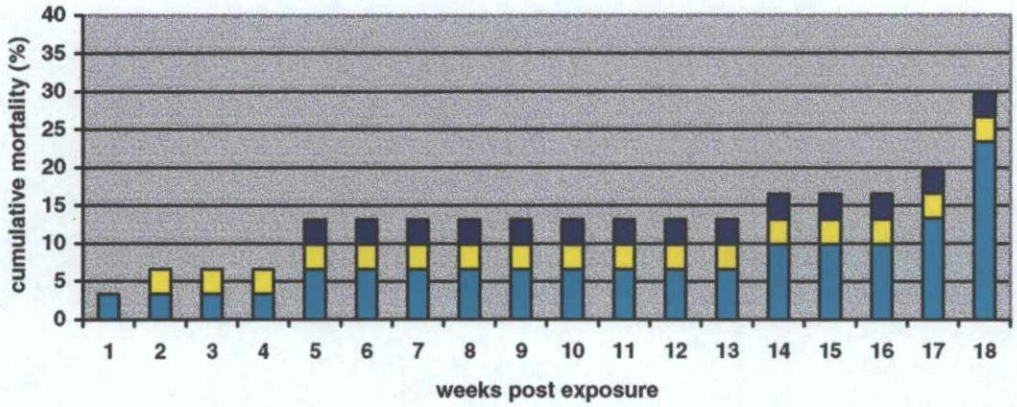

Figure 25. Cumulative mortalities for pre-recruit oysters in tank 2 exposed to 10^2 *B. exitiosus* via the water. Light blue = not infected, Yellow = light *B. exitiosus* infection (grades 1-2), Dark blue = heavy *B. exitiosus* infection (grades 4-5).

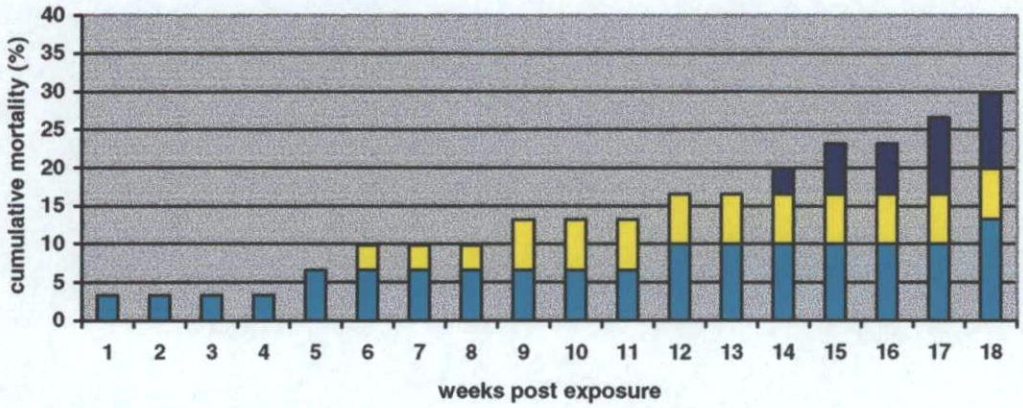

Figure 26. Cumulative mortalities for pre-recruit oysters in tank 3 exposed to 10^3 *B. exitiosus* via the water. Light blue = not infected, Yellow = light *B. exitiosus* infection (grades 1-2), Dark blue = heavy *B. exitiosus* infection (grades 4-5).

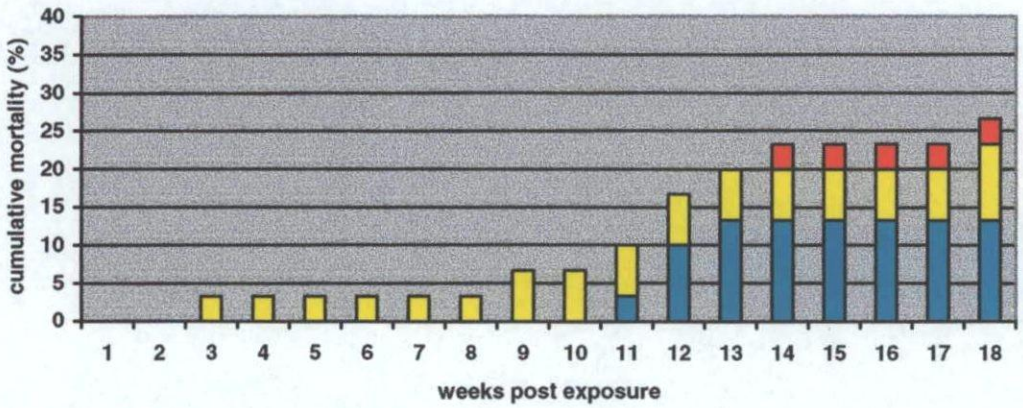

Figure 27. Cumulative mortalities for pre-recruit oysters in tank 4 exposed to 10^4 *B. exitiosus* via the water. Light blue = not infected, Yellow = light *B. exitiosus* infection (grades 1-2), Red = moderate *B. exitiosus* infection (grade 3).

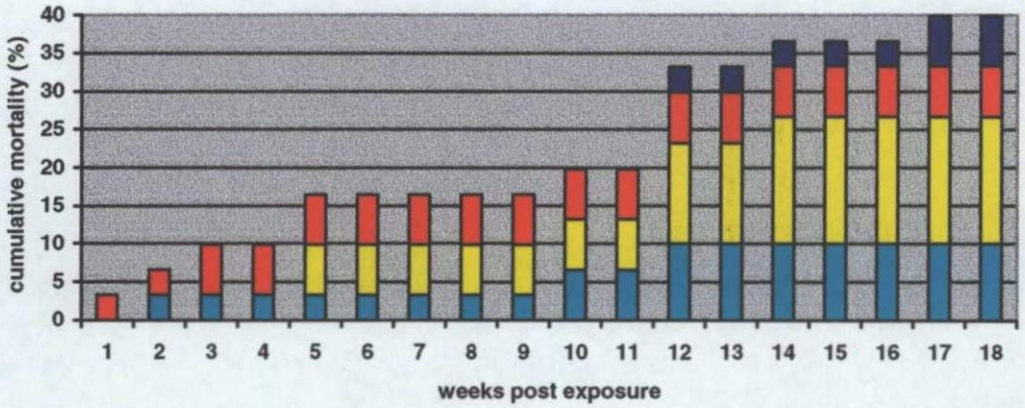

Figure 28. Cumulative mortalities for pre-recruit oysters in tank 5 exposed to 10^5 *B. exitiosus* via the water. Light blue = not infected, Yellow = light *B. exitiosus* infection (grades 1-2), Red = moderate *B. exitiosus* infection (grade 3), Dark blue = heavy *B. exitiosus* infection (grades 4-5).

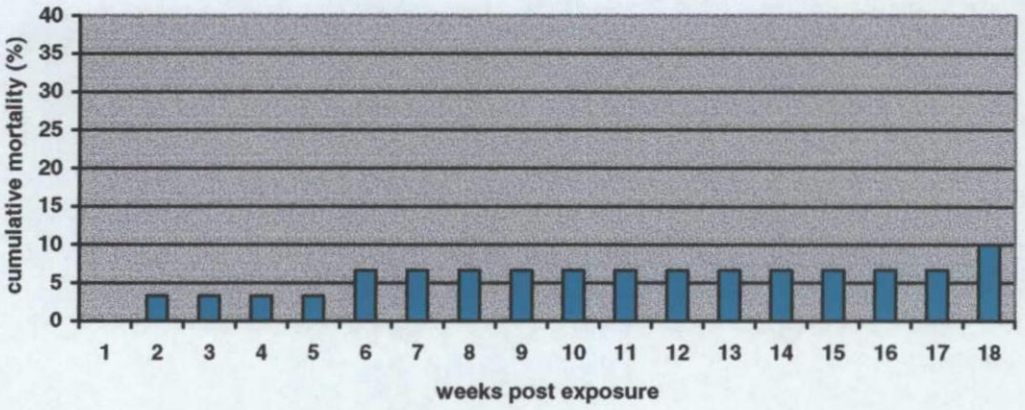

Figure 29. Cumulative mortalities for control pre-recruit oysters in tank 6. Light blue = not infected.

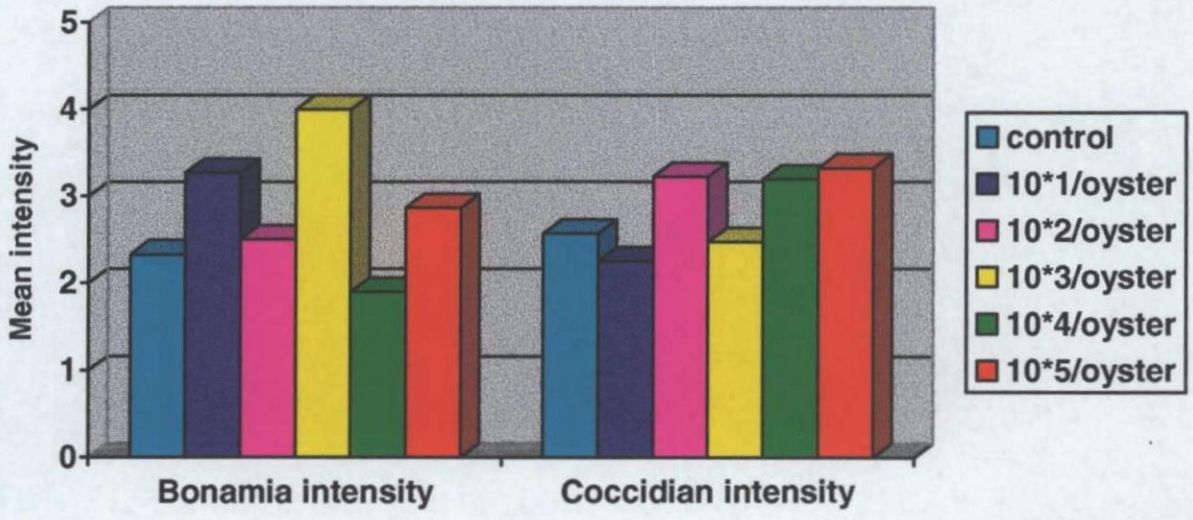

Figure 30. Mean intensity of *B. exitiosus* and an unnamed coccidian parasite in pre-recruit oysters exposed via the water to either 10^1 (dark blue), 10^2 (pink), 10^3 (yellow), 10^4 (green), or 10^5 (red) purified viable *B. exitiosus*, and control oysters (light blue) exposed to clean seawater.

11. Conclusions

The low temperature (8°C) replicate of objective 8 was not completed due to the need to modify the original experimental design in light of the results obtained from the high temperature (15°C) replicate. Modification of the original experimental design is required because the results of the Objective 8 and 10 experiments conducted in unfiltered seawater at Greta Point were confounded by the presence of *B. exitiosus* in both recruit and pre-recruit-sized oysters collected from the wild and from oyster farms. Experimental oysters were also exposed to additional *B. exitiosus* via the water supply and via mortalities of other oysters which died of Bonamiosis during the experiments. Before the outstanding objective 8 experiments (or any other experiments for that matter) are undertaken, revised experimental protocols are recommended to ensure:

1. The experiments are conducted using *B. exitiosus*-free oysters, either pre-recruit cultured oysters from the Marlborough Sounds (after screening for *B. exitiosus* by statistical testing at the 5% disease incidence level), or if recruit sized oysters are required, only after a supply of *B. exitiosus*-free recruit sized oysters is located by statistical testing at the 5% disease incidence level;

2. That the experiments are conducted in a filtered water supply (which would entail feeding oysters algae for the duration of the experiments), to exclude infective stages from the water supply; and

3. Experimental oysters should be held in individual containers to ensure they are exposed to no other sources of *B. exitiosus* during the experiment (including from oysters dying in the same treatment).

With the benefit of hindsight, the low temperature replicate for the objective 8 experiment, if conducted in its originally proposed form, would be unlikely to provide reliable information because of the confounding effect of *B. exitiosus* in the water supply. Furthermore, the original experimental design may not generate enough data because only two temperatures (8°C, 15°C) were being examined. While these temperatures represent the annual extremes in water temperature in Foveaux Strait, most of the important processes driving the *B. exitiosus* outbreaks in the fishery occur at intermediate temperatures. This would strongly suggest the need to generate data for the course of infection over a temperature curve, of a minimum of 3 points, probably 8, 12 and 15°C.

As information from the completed objectives was compiled, it became obvious that it was very important to obtain a reasonable estimate of the potential number of infective stages harboured by moribund oysters. This is because the available data on infectious dose and course of infection are based on the objective 8 and 10 experiments where the actual (or approximate) number of *B. exitiosus* used was known. Knowledge of actual parasite numbers in moribund oysters of various sizes, and infection intensities, could provide valuable information which would allow us to estimate, via the mathematical model, the potential number of *B. exitiosus* infective stages present in a given population of oysters of a particular size and intensity of infection. As time to collect these data was not allocated to any of the previous research objectives, it is not possible to obtain this information at the present time.

A logical extension to the need to quantify the number of infective stages present in moribund oysters, is quantification of the methods used to score *B. exitiosus* in heart smears and histology. There is evidence that the semiquantitative scoring methods used are based on an exponential relationship rather than a linear relationship (i.e. oysters with a score of 4 may harbour 5 to 10 times the number of *B. exitiosus* than do oysters with a score of 3). It is important to determine if

this is the case to ensure the mathematical model is based on accurate assumptions on the number of *B. exitiosus* in oysters of different infection intensities.

It is possible to design a single experiment which would gather the information required to address the issues raised above. By conducting the proposed experiment at 3 temperatures, the unfinished low temperature replicate of objective 8 would be made redundant. Because of the need for modified experimental conditions to eliminate the threat of confounding sources of *B. exitiosus*, however, this proposed work would need to be done in a filtered water supply at the Mahanga Bay aquaculture facility. Because of the logistical requirements for conducting work at this facility, together with the need for extra staff time to set up more elaborate experimental designs using individual oysters, feeding of the oysters, and processing the additional histological material required to determine how the semiquantitative infection intensity scale relates to actual parasite numbers, the cost of the extra work could not be covered under the existing contract. More details for the revised requirements of the additional epidemiological research outlined above are included in the NIWA document outlining recommended research priorities for the Foveaux Strait Oyster Fishery 2002-03.

If these additional data are obtained, a preliminary infection model can be formulated and run to examine whether mathematical modelling of *B. exitiosus* outbreaks can provide useful information for the management of oyster populations in the Foveaux Strait fishery, and where future research priorities for study of the epidemiology of *B. exitiosus* should lie.

12. Publications

Two scientific papers based on some of the results from these experiments have been submitted to international journals. They are currently in the review process and none have been published to date.

Diggles, B.K., Cochennec-Laureau N., Hine P.M. Comparison of diagnostic techniques for *Bonamia exitiosus* from flat oysters *Ostrea chilensis* in New Zealand. *Aquaculture.*

Hine P.M., Diggles B.K., Parsons M., Pringle A., Bull B. The effects of stressors on the dynamics of *Bonamia exitiosus* Hine *et al.* 2001 infections in flat oysters *Ostrea chilensis. Journal of Fish Diseases.*

13. Data management

Hard copies of raw data are stored in the diseases laboratory, NIWA Greta Point. Electronic data are stored in the O drive at NIWA Greta Point. Data sets include details of numbers of oysters examined for each treatment in each objective, and the sex, survival status, *B. exitiosus* status and other health indicators of each oyster. Data are stored in Excel files.

References

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Appendix 1.

Protocol used for *in–situ* hybridization of *Bonamia exitiosis* in Foveaux Strait oyster sections (modified from Cochennec et al. 2000).

PREPARATION

Fix oysters in Davidson's fixative or 10% formalin in filtered seawater for no longer than 48 hours before dehydrating and embedding oysters into wax. Cut sections 5 µm thick and place them onto slides manually coated with 3–Amino propyltriethoxysilane (APES, Sigma).

DAY 1 Make sure you have enough probe for the slides (3 to 5 µl/slide), proteinase K and hybridisation buffer.

0. Place slides in oven at 42° C and heat overnight to remove most wax

1. Dewax slides : 2 x 10 minutes in xylene
   2 x 10 minutes in 100% ethanol

2. add 200 µl of proteinase K in TE buffer per slide and incubate at 37°C (oven), no longer than 30 minutes in moist chamber (use 100µg/ml proteinase k diluted in TE buffer , stored in freezer)

3. dehydrate slides – 1 minute 95% ethanol
   1 minute 100% ethanol

4. place a mixture of 3 to 5 µl of labelled PCR probe* + 50 µl hybridisation buffer onto section, cover with coverslip (Hybaid Easi Seal, large)

5. Denature at 94 to 95°C for 3 minutes 9

6. Place on ice for 1 minute

7. Incubate slides **overnight** at 42°C in a moist chamber (oven). Also incubate 0.4 x SCC.

DAY 2. Make sure you have enough buffers (SSC, DIG 1,2,3), fresh xylene and 100% ethanol.

8. Next day wash slides 5 minutes in 2 x SSC, then another 5 minutes in 2 x SSC

9. Then wash at 42°C in 0.4 x SSC for 10 minutes

10. Place slides for 1 minute in DIG 1 buffer

11. Block tissue section with 200µl of DIG 2 buffer for 30 minutes at room temperature.

12. Place slides for 1 minute in DIG 1 buffer

13. Cover slides with 200µl of diluted a–DIG–conjugate 1 (diluted 1:500 in DIG 2 buffer) and incubate for 1 hour at room temperature .

14. Wash twice for 5 minutes each in DIG 1 buffer to avoid background labelling

15. Place the slide in DIG 3 buffer for 10 minutes

16. Place 200µl of diluted NBT/BCIP (20 µl to 1 ml of DIG 3 buffer, or for 40 slides, 160 µl in 8 ml) onto the section and incubate at room temperature IN THE DARK for 10 to 60 minutes.

17. Stop staining by washing in distilled water. STOP here if cannot stain and mount immediately

18. Stain in 0.5% bismark brown (made in 30% ethanol) for 1 to 2 minutes

19. Dehydrate twice in 95% ethanol, twice in 100% ethanol (**must be 100%**), then 2 washes in Xylene before mounting directly under coverslips using Eukit or similar mountant.

***Preparation of the PCR labelled probe**

1. Extract *B. exitiosus* DNA from heavily infected oyster (proteinase K digestion overnight in water bath at 37°C, then phenol chloroform extraction)
2. Amplify approx. 350 bp region of *B. exitiosus* 18S rDNA using PCR with primers BO (5' CAT TTA AATT GGT CGG GCC GC 3') and BOAS (5' TCT GAT CGT CTT CGA TCC CC 3') after adding 2.5 µl of dUTP–DIG (Boehringer Mannheim, 1573 152) label per 50 µl reaction.

PCR reagents:		Reaction conditions:
PCR Master Kit	50 µl	94°C 3 minutes
Mg CL_2 (25mM)	4 µl	then 30 cycles of
DIG dUTP (25 mM)*	5 µl	94°C for 1 min
BO	2 µl	55 °C 1 min
BOAS	2 µl	72°C 1 min
DNA	2 µl	then to 72°C 10 minutes
Water	35 µl	then hold at 12°C
Total	100 µl	

* (use 2 tubes, add DIG label to 1 tube, run second tube without label as negative control)

Appendix 2.

Calculations for determination of epidemiological definitions (after Fegan 2000).

A hypothetical test used on n = 30 oysters found 19 oysters positive for *Bonamia* (a + b), including 2 false positives (b) (i.e. 17 true positives, a), and 11 negative oysters (c + d), including 4 false negatives (c) (i.e. 7 true negatives, d). The gold standard test found 21 oysters positive for *Bonamia* (a + c) and 9 oysters negative (b + d).

These results can be set out as follows:

	Standard +	Standard –	
Test +	a	b	a + b
Test –	c	d	c + d
	a + c	b + d	a+ b + c + d = n

For this example,

	Standard +	Standard –	
Test +	17	2	19
Test –	4	7	11
	21	9	n= 30

Sensitivity: (proportion of oysters with *Bonamia* which test positive – proportion of true positives)

Sensitivity = (a / (a + c))*100 example, 17/21 * 100 = 81% sensitivity

Specificity: (proportion of oysters without *Bonamia* which test negative – proportion of true negatives

Specificity = (d / (b + d))* 100 example , 7/9 * 100 = 77.8 % specificity

Positive predictive value (PPV): – the probability that an oyster returning a positive test actually has *Bonamia*)

PPV = (a / (a + b))* 100 example, 17 / 19 * 100 = 89.5

Negative predictive value (NPV): – the probability that an oyster which returns a negative test actually does not have *Bonamia*).

NPV = (d / (c + d))* 100 example, 7 / 11 * 100 = 63.6

Apparent prevalence (prevalence determined by test in question)

Apparent prevalence = ((a + b) / n) *100 example, (19/30) *100 = 63.3 %

True prevalence (prevalence as determined by gold standard)

True prevalence = ((a + c) / n) * 100 example, (21/30) * 100 = 70 %

Kappa statistic: a measure of the agreement of a test with a benchmark "gold standard" test

Kappa = (OA–EA) / (1 – EA) example K = (0.8 – 0.553) / (1 – 0.553) = 0.552, moderate agreement

When OA = (a + d) / (n), and EA = ((a + b)/n) * ((a + c)/n) + ((c + d)/n) * ((b + d)/n)

Appendix 3.

Revised *B. exitiosus* purification protocol (based on Miahle et al. 1988).

Prepared prior to undertaking purification:

Reagents:

1.5L 0.22µm **filtered seawater (FSW)**

100mL 20% **sucrose**/FSW (25g/100mL)

1L filtered seawater with 1% **Tween 80** (FSWT)
250mL 20% sucrose/FSWT (w/w) (62.5g/250mL)
200mL 40% sucrose/FSWT (w/w) (133.3g/200mL)

100mL isosmotic **Percoll** by adding 0.4M **NaCl** (2.7g/100g) (2.97g/100mL)
20mL 30% (v/v) Percoll in FSWT (i.e. 6:14)
20mL 40% (v/v) Percoll in FSWT (i.e. 8:12)
20mL 50% (v/v) Percoll in FSWT (i.e. 10:10)
20mL 60% (v/v) Percoll in FSWT (i.e. 12:8)
20mL 70% (v/v) Percoll in FSWT (i.e. 14:6)

Equipment:

500mL beakers	Sieves 300–, 75–, 25µm mesh
200mL beakers	50mL graduated centrifuge tubes with lids
100mL beakers	15mL graduated centrifuge tubes with lids
Haemocytometer	1mL, 5mL, 10mL, 25mL pipette tips
Syringe with long needle	Peristaltic pump
Blender (Polytron)	Centrifuge

1.1 *B. EXITIOSUS* PURIFICATION

Rinse 3 heavily infected oysters with FSWT
Pool oysters (without adductor muscle) in a 500mL beaker with 100mL FSWT
Homogenize oysters until no lumps visible using polytron (blender)

Sieve the mixture through a succession of fine (300–, 75–, 20µm) mesh filters
(Use the back of a spoon to push the mixture through the mesh)

Dispense homogenate equally into five 50mL plastic centrifuge tubes (~20mL each)

2. CENTRIFUGE AT 2000 G AND 8°C FOR 30 MINUTES

Discard the supernatant
Resuspend the pellet in each tube by adding 10mL FSWT. (5x1mL + 5mL per tube)
Pool the suspension in a beaker and gently re–homogenize

Dispense 25mL 20% sucrose/FSWT in each of five 50mL centrifuge tubes
Layer 10mL of homogenate on top of each tube of sucrose using **peristaltic pump**

3. CENTRIFUGE AT 2000 G AND 8°C FOR 30 MINUTES

To SEVEN 50mL centrifuge tubes add 25mL 40% sucrose/FSWT
Then add 12mL 20% sucrose/FSWT using **peristaltic pump**

Discard the supernatant
Add 1mL FSWT to each tube and resuspend
Add 6mL FSWT to each tube and resuspend
Pool suspension in one tube

Gently layer 5mL of suspension of top of each tube (the 7 tubes with sucrose gradient) using **peristaltic pump**

4. CENTRIFUGE AT 2000 G AND 8°C FOR 30 MINUTES

Can start Percoll gradient now (see below*)
Collect the band of material at the 20/40% interface using a syringe
Pool in one tube and slowly dilute to 50% (v/v) with FSWT

5. CENTRIFUGE AT 2000 G AND 8°C FOR 30 MINUTES

* In each of SIX 15mL centrifuge tubes prepare a discontinuous Percoll/FSWT gradient. Add 2.5mL 70% Percoll/FSWT to each tube. Using **peristaltic pump** add 2.5 mL of 60% then 50% then 40% then 30% to each tube

Discard the supernatant and wipe the inside upper edge of tube
Re–suspend the pellet in 6mL **FSWT**

Add 1mL of suspension to each of the six tubes with Percoll/FSWT gradient

6. CENTRIFUGE AT 2000 G AND 8°C FOR 30 MINUTES

Collect the bands at the 50/60 and 60/70% interfaces using a syringe and pool in one tube
Dilute to 50% (v/v) with **FSW** (no Tween)

Add 10mL **20% sucrose/FSW** to SIX 15mL centrifuge tubes
Layer 1mL suspension onto each tube using **peristaltic pump**

7. CENTRIFUGE AT 2000 G AND 8°C FOR 30 MINUTES

Remove supernatant using a micropipette – leave a small amount of liquid at the bottom
Add 1mL **FSW** (no Tween) to each tube and resuspend
Pool in one 15mL centrifuge tube

8. CENTRIFUGE AT 2000 G AND 8°C FOR 30 MINUTES

Discard the supernatant and re–suspend in 1mL FSW.
Dilute 3µl with 3µl trypan blue in filtered seawater to count viable parasites on a haemocytometer (Malassez cell)