

***BONAMIA OSTREAE* IN THE NATIVE OYSTER  
*OSTREA EDULIS***

**A REVIEW**

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## FOREWORD

Ireland has a long history of producing and harvesting native flat oysters, *Ostrea edulis*. At the start of the nineteenth century, almost every bay and harbour, around the coast – including Carlingford, Malahide, Sutton, Dublin, Arklow, Wexford, Youghal, Cork, Kinsale, Kenmare, Tralee, the Shannon estuary, north Clare, Galway, Kilkieran, Ballinakill, Clew Bay, Achill, Blacksod and around Donegal – had abundant beds of native oysters. Intensive dredging to meet the demands of the markets in Dublin and England depleted the stocks, so that in 1845 the government passed legislation to permit the formation of private oyster beds to improve the ailing stocks. According to the book on “Shellfish & Shellfisheries of Ireland” (Wilkins, 2004) Irish stocks began to collapse between 1850 and 1860. By the second half of the twentieth century, only the beds of Tralee Bay, Galway Bay and Clew Bay were still yielding a good return for local fishermen.

The arrival of the oyster parasite *Bonamia ostreae* in the mid 1980s was an additional blow to the Irish native oyster stocks. This report sets out to document the spread and the impact of the *Bonamia ostreae* parasite in Irish bays since the 1980s. The Marine Institute has commissioned two of the leading scientists in shellfish health, Prof. Maire Mulcahy & Dr Sarah Culloty of University College Cork to compile this report, in order to increase awareness of the importance of pro-active shellfish health management. We wish to express our thanks to the authors and to external reviewers, including Mark Norman and Dr. Iarfhlaith Connellan, for their expert input.

Members of the shellfish industry and State agencies (Department of Marine and Natural Resources, Sea Fisheries Protection staff, Fisheries Boards, Marine Institute, BIM and Taighde Mara ) can use this report as a reference document on *Bonamia* control, research and risk management. We welcome feedback on the Recommendations. If these lessons are learned and put into practise, Ireland can still maintain its status of being free from Marteiliosis throughout the coast and can protect the *Bonamia*- free status of bays such as Tralee and Kilkieran.



## CHAPTER 1. WHAT IS BONAMIOSIS?

Bonamiosis is a disease of oysters, caused by a group of protistan parasites of the genus *Bonamia*. Species include *Bonamia ostreae* (Pichot *et al.*, 1980) and *B. perspora* (Carnegie *et al.*, 2006) in the Northern Hemisphere. A number of species of flat oyster are known to be susceptible to *B. ostreae*: the flat oyster, *Ostrea edulis* L. (Grizel *et al.*, 1983), and *Ostrea conchaphila* (= *O. lurida*), *Ostrea angasi*, (Bougrier *et al.*, 1986; Bucke and Hepper, 1987), *Tiostrea chilensis* (Hutton) (Bucke *et al.*, 1984), and *Ostrea puelchana* (Pascual *et al.*, 1991). To date, the oyster *Ostrea lurida* on the east coast of the United States is the only species known to be infected by *B. perspora* (Carnegie *et al.*, 2006). An unidentified *Bonamia* sp has also been observed in the introduced *C. angulata* on the east coast of the U.S. (Burreson *et al.*, 2004) and in the Suminoe oyster *Crassostrea rivularis* reared in France (Cochennec *et al.*, 1998).

In the Southern Hemisphere, a number of *Bonamia* species are present (Dinamani *et al.*, 1987). *Bonamia exitiosa* infects *Tiostrea chilensis* in New Zealand (Hine *et al.*, 2001; Berthe and Hine, 2003; Bower and McGladdery, 2003), and *B. roughleyi* (formerly *Mikrocytos roughleyi*) parasitises *Saccostrea glomerata* and is the causative agent of Australian winter disease (Farley *et al.*, 1988; Cochennec-Laureau *et al.*, 2003a; Carnegie and Cochennec Laureau, 2004). *Bonamia*.sp. has been found in *T. chilensis* in Chile, and in *Ostrea puelchana* in Argentina (Campalans *et al.*, 2000; Kroek & Montes, 2005), and can infect *O. angasi* and *O. denselammellosa*, as well as *Crassostrea rivularis* (= *C. ariakensis*), (OIE, 2006; Carnegie and Cochennec-Laureau 2004; [www.pac.dfo-mpo.gc.ca/sci/shelldis/pages/bonosty\\_e.htm](http://www.pac.dfo-mpo.gc.ca/sci/shelldis/pages/bonosty_e.htm) 2006).

## CHAPTER 2. DISTRIBUTION OF BONAMIOSIS WORLDWIDE

*Bonamia ostreae* has spread throughout many countries where flat oyster production occurs. The parasite can cause over 90% mortality among oysters, when initially introduced into a naïve population, and significant economic losses for farmers have occurred as a result. The parasite is believed to have initially been spread by movements of oysters in the 1970's in the United States from California to Maine and Washington and to France and Spain in Europe (Elston *et al.*, 1986; Friedman *et al.*, 1989; Friedman and Perkins, 1994; Cigarría and Elston, 1997). The parasite had initially been observed in California a decade earlier (Katkansky *et al.*, 1969; Elston *et al.*, 1986) (Figures 1 and 2).

*Bonamia ostreae* was initially detected throughout Brittany, France in 1979 (Comps *et al.*, 1980; Grizel and Tige, 1982), Spain and Denmark in 1980 (Van Banning 1985; Figueras, 1991), Fal and Helford Rivers in Cornwall, England in 1982 (Bannister and Key 1982; Bucke *et al.*, 1984; Hudson and Hill, 1991), and it was first recorded in Ireland in 1987 (McArdle *et al.*, 1991). The spread of bonamiosis has continued. Most recent records were in British Columbia, Canada in 2004 (Marty *et al.*, 2006), Morocco in 2005 (<http://www.oie.int/Messages/05112MAR.htm>) and Scotland <http://www.scotland.gov.uk/News/Releases/2006/07/27154609> and Wales in 2006 <http://new.wales.gov.uk/news/archivepress/environmentpress/enviropress2006/1089034/?lang=en>

In the nineteenth century the European oyster industry was large and extensive. In the 1920's the industry suffered its first large scale mortalities (Orton, 1924) from which some areas, particularly in England, did not recover. The French industry did recover however, and thrived until the 1960s when large scale mortalities, – over 90%, were recorded in *O. edulis* in Aber Wrach in Brittany. The parasite *Marteilia refringens* was diagnosed in dying oysters, and within three to four years most of the oyster growing regions within Brittany were infected with this parasite. Production figures from 1960-1970 within France were 20,000 tons per annum, but by the early 1970s the effects of *Marteilia* were being felt, with production dropping to 10,000 tons in 1973 and to 8,400 tons in 1975. With the introduction of a second parasite *B. ostreae* was also causing mass mortalities in 1979, the industry has been unable to recover to anything like the production figures attained in the 1960's. Overall European production of cultured flat oysters fell from 29,595 tons in 1961, to 5921 tons in 2000, due to the epizootics caused by *B. ostreae* and *Marteilia refringens* (F.A.O. Fisheries Department, 2002). Due to the impact of diseases and a consequential shift to the rearing of the Pacific cupped oyster (*Crassostrea gigas*), European flat oyster production has remained low throughout the decade 1993-2002; output peaked in 1996 (7,996 tonnes) but became more stable (6000-7000 tonnes) from 2000 to 2002. In 2002, 67 percent of the production was in Spain (4,565 tonnes) and 24 percent in France (1,600 tonnes). Ireland and the UK were the only other countries that produced more than 200 tonnes in 2002.

The production of the European flat oyster constituted less than 0.2 percent of the total global production of all farmed oyster species in 2002. The bulk of production (97.7 percent) came from the rearing of the Pacific cupped oyster, *Crassostrea gigas*. However, the value of farmed *O. edulis* production in 2002 was US\$ 24.3 million; thus its culture remains an important sector in the limited areas where it is reared ([http://www.fao.org/fi/website/FIRetrieveAction.do?dom=culturespecies&xml=Ostrea\\_edulis.xml](http://www.fao.org/fi/website/FIRetrieveAction.do?dom=culturespecies&xml=Ostrea_edulis.xml))

It is considered that the spread of *B. ostreae* has been mainly due to the movements of infected oysters to new ongrowing areas, or to inadvertent movements of infected oysters with other shellfish. The spread of the parasite has also been attributed to the use of equipment in areas infected with the parasite e.g. to Lake Grevelingen in the Netherlands (Van Banning, 1991). Other mechanisms, suggested as means of transmission of *B. ostreae*, include fouling on boat hulls (Howard, 1994).



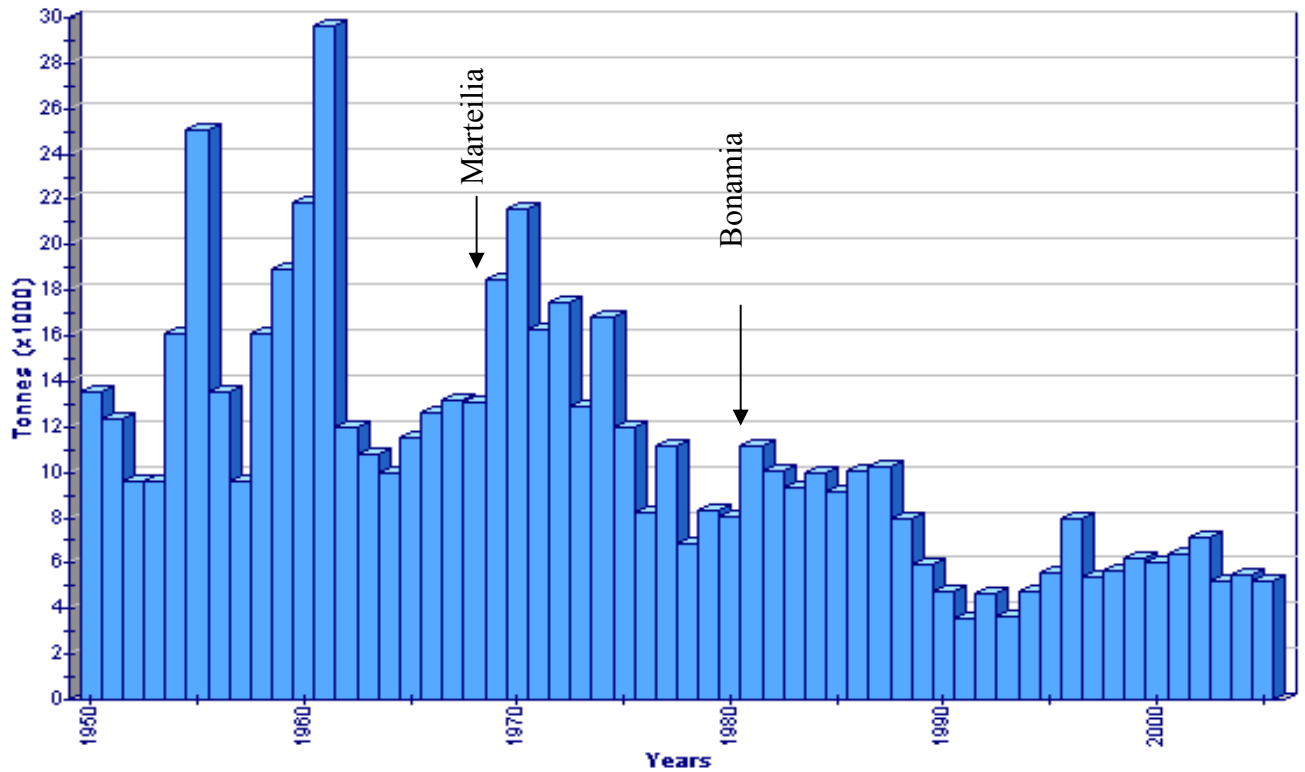


Figure 1. Production figures for *O. edulis* worldwide from 1950 - 2005.

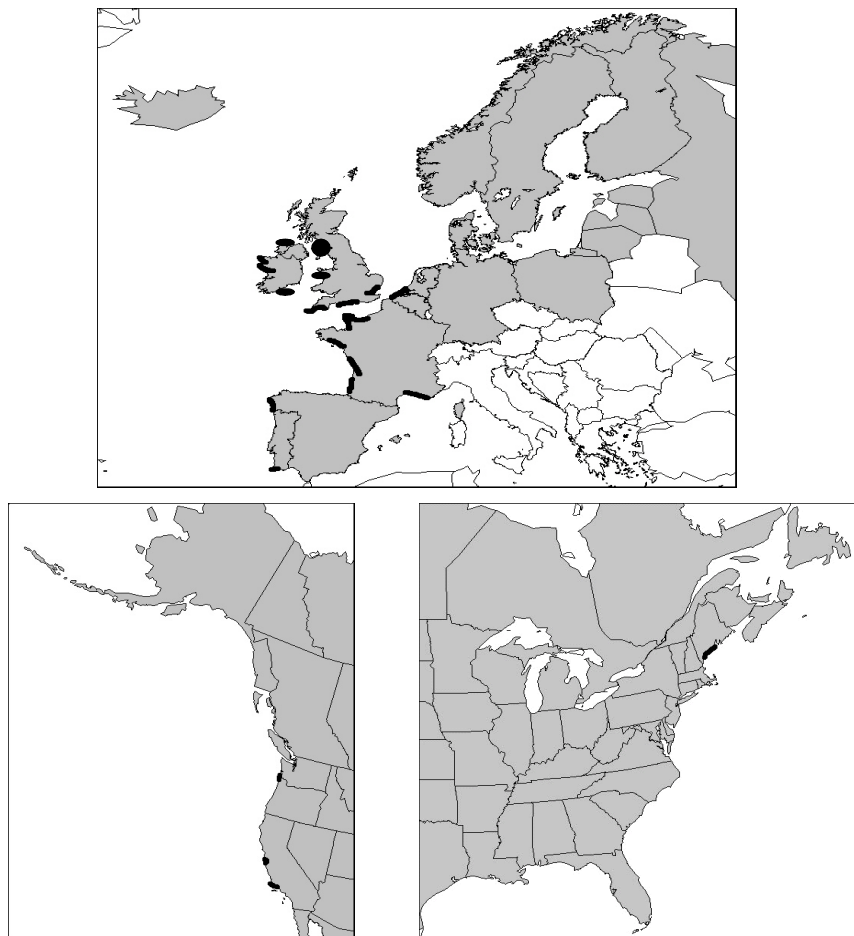
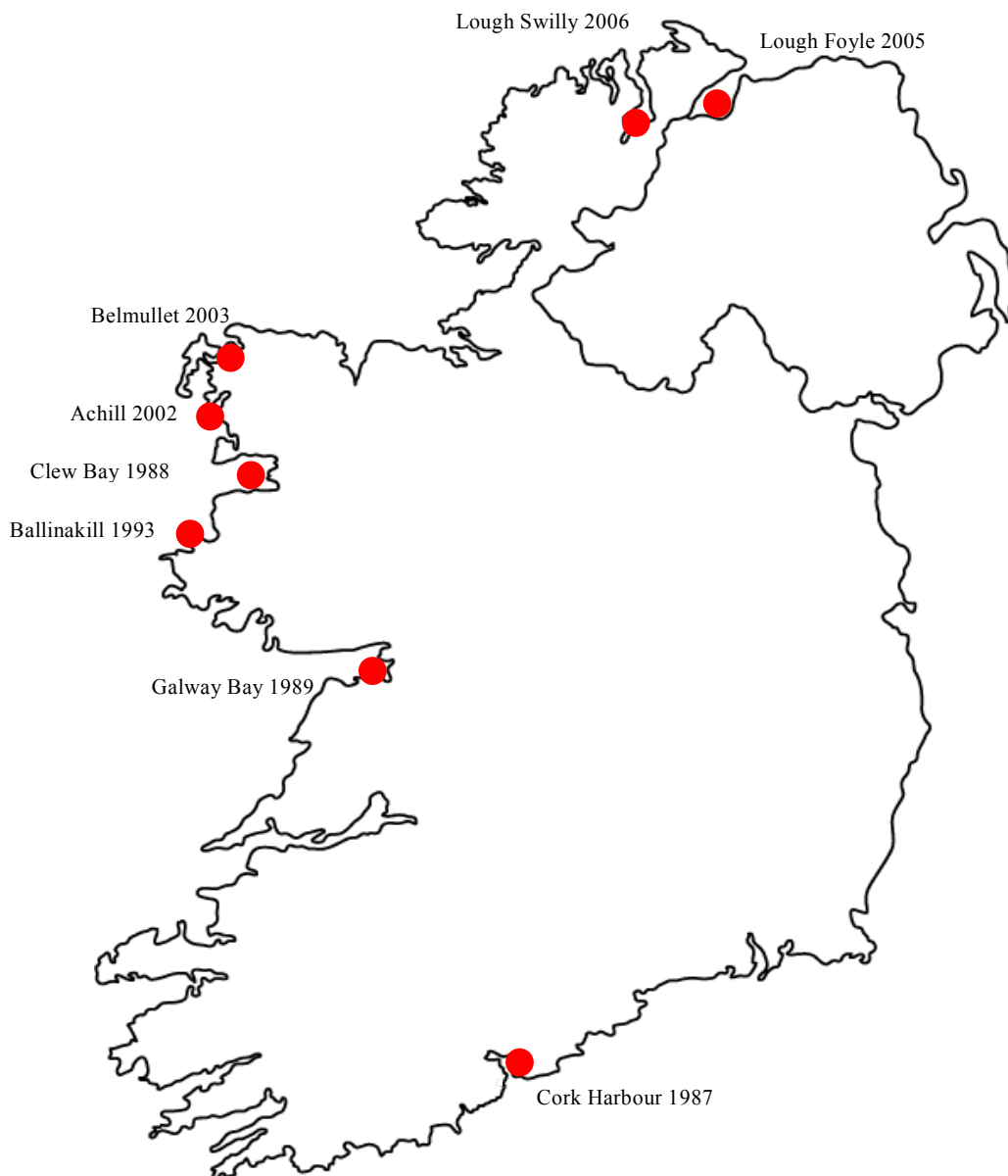


Figure 2. Current distribution of *B. ostreae* within Europe and North America. ([www.ices.dk/.../fishdiseases/images/map8\\_5.gif](http://www.ices.dk/.../fishdiseases/images/map8_5.gif))

### CHAPTER 3 . THE SPREAD OF BONAMIOSIS IN IRELAND

*Bonamia ostreae* has been present in Irish oyster stocks since the 1980s (Figure 3). Oyster stocks in Rossmore, in the North Channel of Cork Harbour, suffered increasing mortalities from 1984, and were affected by mortalities of over 90% of 4+ aged oysters in 1987 (McArdle *et al.*, 1991). The cause was diagnosed as bonamiosis, the first record of this disease in Ireland (McArdle *et al.*, 1991). Exactly how or when the parasite had been initially introduced is unknown. It appears likely that *B. ostreae* had been present in the stocks for at least a couple of years before 1987, and the parasite was subsequently recorded in Rossmore oysters which had been frozen in 1986 (Rogan *et al.*, 1991).



**Figure 3.** Distribution of *B. ostreae* and its progression within Irish oyster growing regions since its introduction in the 1980s.

### **Clew Bay**

In 1988, *B. ostreae* was diagnosed in a consignment of wild oysters from Clew Bay, exported to France. No mortalities were observed at that time and it was a number of years before follow-up surveys of Clew Bay oysters confirmed the presence of *B. ostreae* at low levels (Mc Ardle *et al.*, 1991).

### **Galway Bay**

In 1989, *B. ostreae* was diagnosed in Galway Bay in oysters, which had been relayed from natural beds in the Bay to more sheltered inlets, where conditions were most favourable. While no mortality was apparent in the rest of Galway Bay at the time, 70%-80% losses occurred in localised relaying areas. In 1991, bonamiosis was observed in five new sites within the Bay, though at low prevalence and with no observed mortality (Mc Ardle *et al.*, 1991).

### **Achill, Co Mayo**

In 1994, routine statutory testing of oysters for notifiable diseases in the natural oyster beds of Achill Sound detected one *B. ostreae* – positive oyster out of 450. Spring and autumn samples, taken over the next seven years, were all negative. In spring 2002 however, low intensity *B. ostreae* was detected in two (6.6%) of an oyster sample of 30, from one of four natural beds in Achill Sound, Co. Mayo (Lyons Alcantara *et al.*, 2004). All four oyster beds were sampled in 2003 and bonamiosis was confirmed in three of them, at prevalences ranging from 13% to 38% and intensities from Class 1 to Class 4 (the highest). The remaining oyster bed was negative (Lyons Alcantara *et al.*, 2004). In the following year, 2004, this latter bed was found to have a 20% infection (n= 30), with intensities ranging from Class 1 to Class 4.

### **Blacksod Bay, Co. Mayo**

The natural oyster stock of the neighbouring Blacksod Bay, where oyster samples from 1994 had been consistently negative, was again sampled in 2003. One of 297 oysters was diagnosed as positive for *B. ostreae*, with an intensity of Class 1. This was confirmed by the EU Reference Laboratory for Mollusc Diseases. A further sample of 150 oysters, taken a month later from the same area as the infected oyster, was negative. However, the routine monitoring of the Blacksod Bay beds in spring 2004 showed up one Class 3- infected oyster out of a sample of 150.

### **Lough Foyle and Lough Swilly**

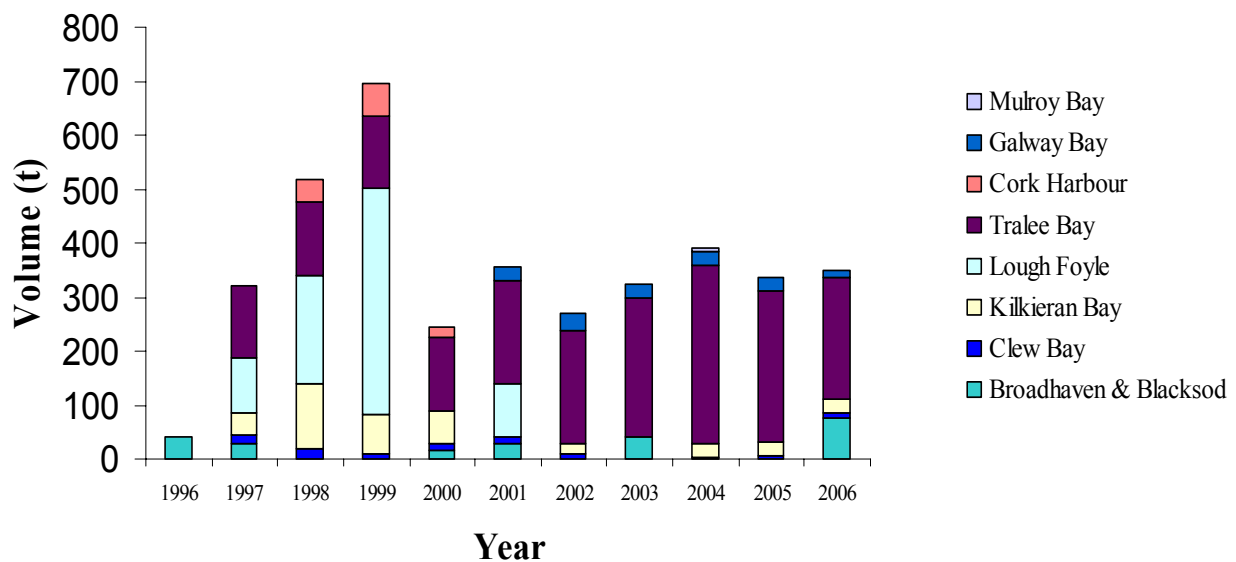
More recently, oysters in Lough Foyle and Lough Swilly have been found positive for *B. ostreae*, in 2005 and 2006 respectively. Until the discovery of *B. ostreae* in Lough Foyle in 2005, the entire coastline of Northern Ireland had approved zone status for both *B. ostreae* and *M. refringens*. In the spring of 2005 one of the routine monitoring samples of oysters from one of the oyster beds in Lough Foyle came up positive for *B. ostreae*, at a prevalence of 43 %; the other two samples were negative. A follow up sample, screened by heart imprints had a prevalence of 6 %. Lough Foyle oysters had been tested twice a year for the previous twelve years in compliance with statutory testing, and had been consistently negative for *B. ostreae*. Responsibility for Lough Foyle lies with both the Northern and Southern Irish authorities, since the Lough lies in both jurisdictions, and is overseen by the Loughs Agency. The spring 2005 samples of oysters, taken from the eastern (Northern Ireland) side of the Lough, were negative for *B. ostreae*. The autumn sampling of Lough Foyle involved screening by histology by northern authorities and screening by cellular imprints by southern authorities. Oysters from five areas on the western and middle of the Lough, examined by the Marine Institute, showed one positive oyster out of one sample of 150, using imprints, and 7 positives out of the same sample, using PCR; the samples from the remaining four areas were negative. The PCR products were confirmed as *B. ostreae* by sequencing. Samples from six areas on the eastern and middle of the Lough, examined by the Agri-Food & Biosciences Institute (AFBI), were all

negative by histology, but a total of six positives from three of the samples tested positive by PCR. It was concluded that *B. ostreae* is distributed across the Lough at low prevalence. An investigation into the source of the *B. ostreae* infection concluded that the most likely possible source was an unauthorised movement of mussels from a non-approved zone in the west of Ireland to Lough Foyle, which is reported to have contained oyster spat. However movement of boats or equipment could also have been a contributing factor (Marine Institute *et al.*, 2006).

Following notification of the EU by Irish authorities, north and south, and the confirmation of the diagnosis of *B. ostreae* by the Community Central Reference Laboratory, Lough Foyle is no longer categorised as bonamiosis-free. Currently Tralee Bay, Co. Kerry and Kilkieran Bay, Co. Galway are the two main oyster growing regions within Ireland free of the parasite.

### Native Oyster Production

By the 1970s only four of the twenty four native oyster fisheries in existence in 1900 still remained – Tralee Bay, Clarenbridge, Clew Bay and Blacksod Bay (Barry, 1980). Of these areas, Tralee has consistently been the most productive with up to 680 tons per annum being produced in the mid 1970’s. During this period approximately 150 tons per annum were produced in Clarenbridge and Clew Bay and 30 tons per annum in Blacksod Bay (Barry, 1980). However, by the early 1990’s production for the whole of Ireland was recorded at 420 t per annum and this had increased to 696 t in 1999, with Tralee and Lough Foyle being the most productive regions (Figure 4). However, following a dramatic decrease in production levels to 266 t in 2000 production had increased to 350 t in 2004 (Figure 4). Nevertheless, in 2004, flat oyster production made up only 1% of the total volume and 3.8% of the value of shellfish production in Ireland (Browne and Deegan, 2006).



**Figure 4.** Production figures from Irish oyster beds (1996-2006)(Data courtesy of BIM)

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## CHAPTER 4. CASE STUDIES IN THE SPREAD OF *BONAMIA*

### Cork Harbour

The North Channel, Cork harbour, had been a long established public oyster bed until it went into decline at the beginning of the twentieth century (Blake, 1870; Browne, 1904). Restocking of the beds began in the 1970s, when Atlantic Shellfish Ltd. obtained a fishery order for the beds. The North Channel of Cork harbour runs in an east west direction between Great Island and the mainland (Figure 5). The depth of the beds varies between 1 and 10 m. The Channel bottom is a mixture of mud, silt and old shell. Salinity in the Channel varies from 25.8-34.0 ‰. Temperatures can range from 6 to 20+°C. Atlantic Shellfish Ltd. began to produce *Ostrea edulis* spat in static sea water ponds on Brick Island in the North Channel. Spat, collected on mussel shell cultch, was subsequently relayed at a density varying between 400-700/m<sup>2</sup>. Prior to the summer of 1987, when bonamiosis was initially diagnosed (McArdle *et al.*, 1991), the North Channel supported an estimated population of 13 million oysters (McManus, 1988).

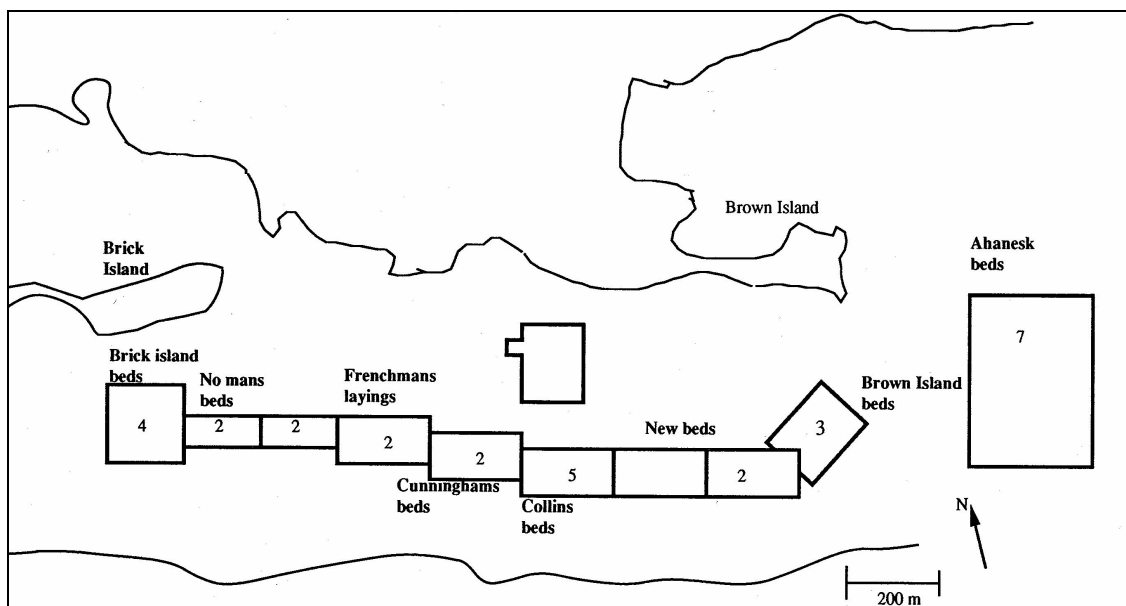
McManus (1988) carried out a study of the beds in 1987 and estimated that the population size on individual beds varied from  $2.2 \times 10^3$  to  $1.5 \times 10^6$ , with the density of oysters varying from 4 to 28 oysters per m<sup>2</sup>. However, the density of oysters prior to the summer of 1986 had been much higher on some beds; e.g. the density on the Collins bed varied from 45 to 131 oysters/m<sup>2</sup>, but after the summer of 1986 the density on this bed had fallen to 0.26 to 7 oysters/ m<sup>2</sup>. McManus attributed these decreases in density to oyster mortalities, due to the presence of *B. ostreae*. In 1988, survival of oysters from spat to three year olds was very low, at best 2.6% and on average 1.5%, due to a combination of spat mortality and the disappearance of cultch through mechanical destruction, biodegradation and movement by currents, whereas the survival of 1984 stock was 11.6%. In the different oyster age groups McManus observed, mortality levels were low up to the 30<sup>th</sup> month, when they rose dramatically to more than 90%. During this study he also noted that the prevalence of bonamiosis increased with age, from 12% in 21 month old oysters on the Brick Island Beds to 51% in 47 month old stock on the Collins Bed (Figure 6). The intensity of infection was also higher in the older animals. Condition of the older animals was also lower than that of younger oysters, which may have been related to the effects of the latter stages of infection. Overall, McManus observed that the most heavily affected beds were in the Collins Bed, which had been the most productive prior to the introduction of the parasite. Some mortality was also attributed to predation by *Carcinus maenas*, but this was in part thought to be due to opportunistic foraging on oysters already weakened by infection (McManus, 1988).

High mortalities were first observed in the summer of 1986 but unusual mortalities had occurred from 1984 (Hugh Jones pers comm.). The parasite was initially diagnosed in March 1987 (McArdle *et al.*, 1991). The mortalities in 1986 had been more severe on the Brick Island Beds, and McManus (1988) postulates that over the summer and winter of that year, the disease spread eastwards along the Channel, with the Collins Bed becoming infected and suffering high mortalities during the summer of 1987. Subsequent work by Rogan *et al.* (1991) found that the parasite was present in oysters that had been sampled in 1986 from the North Channel, and frozen. With a significant level of infection observed in these oysters, it appears likely that the parasite had been present for some time prior to the summer of 1986. As part of an E.C. study, AQ1-272, McArdle carried out an epidemiological study of a number of Irish oyster beds in 1991, including Cork Harbour, where he found that over 45% of market - sized oysters were infected (McArdle *et al.*, 1991).



**Figure 5.** Cork Harbour and the location of the North Channel where *B. ostreae* was initially detected.

Throughout the 1990s infection continued to occur on the oyster beds (Culloty and Mulcahy, 1996) and it is still present (Culloty, *pers. obs.*). However, prevalence and intensity of infection and mortalities have decreased over the years (Culloty et al., 1999; 2001, 2004). Since the parasite was initially diagnosed, selective breeding for resistance has taken place in Cork Harbour. This has taken the form of large scale breeding trials in the spatting ponds, using four to five year old survivors of the disease (Figures 7 and 8). In laboratory and field based trials comparing the susceptibility of the Cork harbour oysters (Rossmore) with Irish and European stocks the Rossmore oysters have performed well (Culloty *et al.*, 2001 and 2004).



**Figure 6.** Map of the oyster beds in the North Channel, Cork Harbour

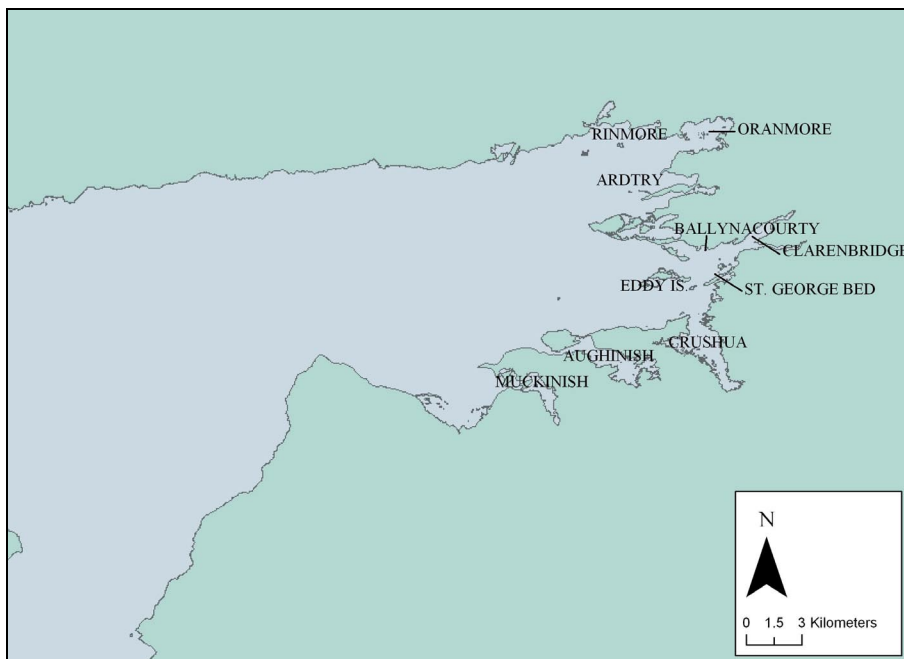


**Figures 7 and 8.** The spatting ponds in the North Channel of Cork harbour.

It appears that many movements of oysters stocks occurred around Ireland in the 1980s. It is not clear if information is available on all these movements, and the basis of the initial introduction of the disease into Cork Harbour cannot be sourced. As the exact date of the initial introduction is unclear, it is not possible to determine how long the parasite had to establish itself, prior to the initial diagnosis. Results of resistance trials for Rossmore oysters have been promising in the last few years, and appear to indicate that a managed breeding program can result in oysters with decreased susceptibility to *B. ostreae* (Culloty *et al.*, 2001 and 2004)

### Galway Bay/Clarenbridge

Within this region the two main oyster growing areas consist of the beds within Galway Bay itself and in the adjacent Clarenbridge beds (Figure 9). The ten oyster beds in the Galway Bay area cover an area of 1,500 hectares, while the Clarenbridge beds cover an area of 400 hectares. In 1971, the Clarenbridge beds in Galway Bay were the second largest public fishery in the country, yielding between 30 and 60 tonnes of oysters each December with a yield of 0.1 oysters/m<sup>2</sup> (Whilde and Edwards, 1970).



**Figure 9.** Map of Galway Bay showing oyster beds.

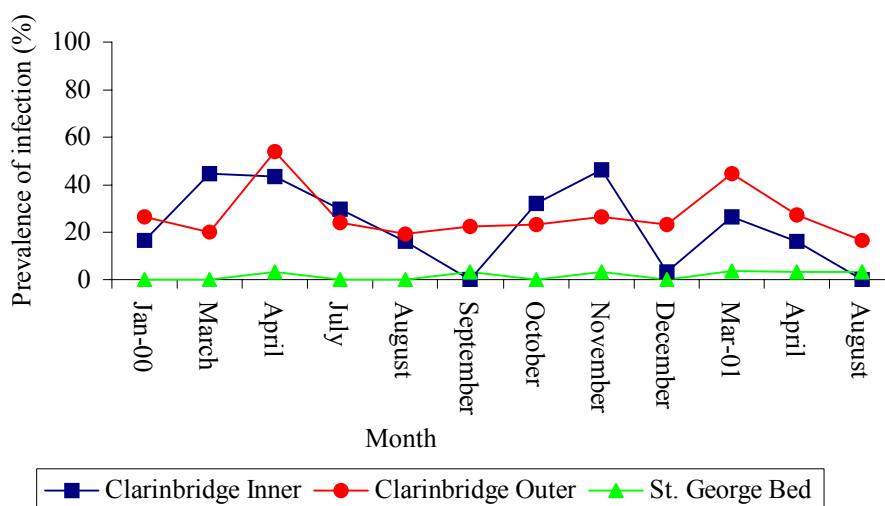
From 1991 to 1994 the seasonal fishery in Galway Bay contributed 50 tonnes of oysters per annum to the overall Irish production of 900 tonnes. However, densities of oysters have



dropped significantly in recent years, due to mortalities resulting from *B. ostreae*. Initial diagnosis of oysters positive for *B. ostreae* occurred within Kinvarra Bay in 1989. Mortalities reached 70-80% and the prevalence of infection was recorded at 50%. In 1991, McArdle, as part of an epidemiological study for an EU project (AQ1-272) found oysters positive for *B. ostreae* within Auhinish, Kinvara and Rincarna Bays. He subsequently found infected oysters in a total of five sites that year (McArdle *et al.*, 1991). The parasite was first recorded in the Clarenbridge beds in June 1991 and in December 1991 oysters from three sites in the centre of the St. George's bed were shown to harbour infection. In 1993, a high incidence of infection was observed in the Clarenbridge oyster beds and adult stocks were severely depleted. In 1997, Beare *et al.* (1998) recorded a low prevalence of infection (6.7–22.2%) in the oyster beds, in a survey carried out in November 1987. In a further survey of the Clarenbridge and St. Georges beds between 2000 and 2001 prevalence of infection varied between 0-54.2% on the Clarenbridge beds and 0-3.5% on the St. George beds, showing a pattern of seasonal variation in prevalence of infection (Figure 10). The reduced infection levels observed in the St. George's bed also warrant some further attention, as densities of oysters on the bed were similar to that in Clarenbridge.

More recently *C. gigas* has been relayed to several beds within the area to compensate for losses in *O. edulis* production. In the last few years approximately 10-15 tons of flat oysters have been produced each year and 180 tons of Pacific oysters (Browne and Deegan, 2006).

In this Fishery overfishing had already depleted stocks, which were further affected by disease. Because of the large size of the beds, a cohesive management plan, to deal with the effects of *B. ostreae*, has been difficult to implement. As fishing continued in the years following diagnosis, older, possibly less susceptible oysters were removed from the beds and were not available to breed. As the population was self sustaining, a mixed population of older, less susceptible and younger, more susceptible oysters were able to breed together, taking a much longer time to build up some degree of resistance in the stocks. Designated areas, where different sized oysters could be grown and selected for resistance, would be required to initiate development of a more resistant stock.



**Figure 10.** Prevalence of infection (%) of oysters from three sites in Galway Bay from January 2000 to August 2001.

### Brittany, France

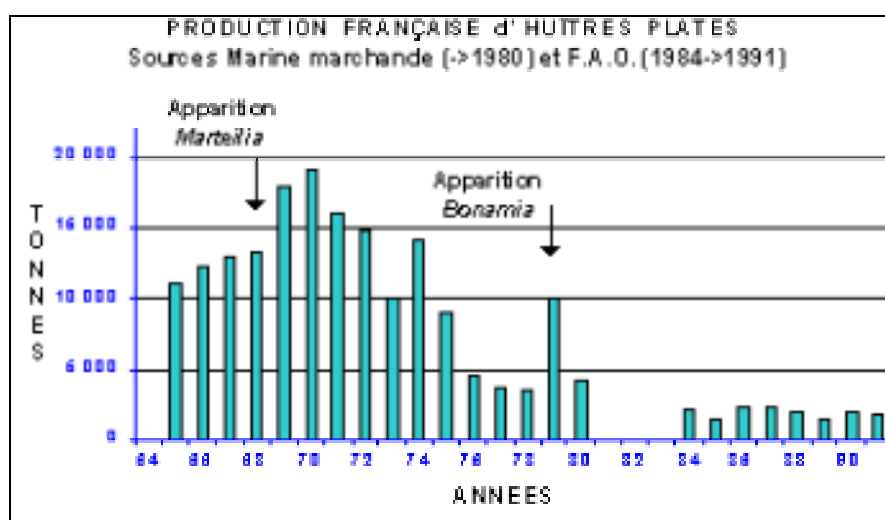
In France, two parasites, *Bonamia ostreae* together with *Marteilia refringens*, have had a severe impact on the production of flat oysters, with 20,000t being produced in 1970, compared to less than 2000t presently (Arzul *et al.* unpubl data). Cumulative losses, due to the diseases in



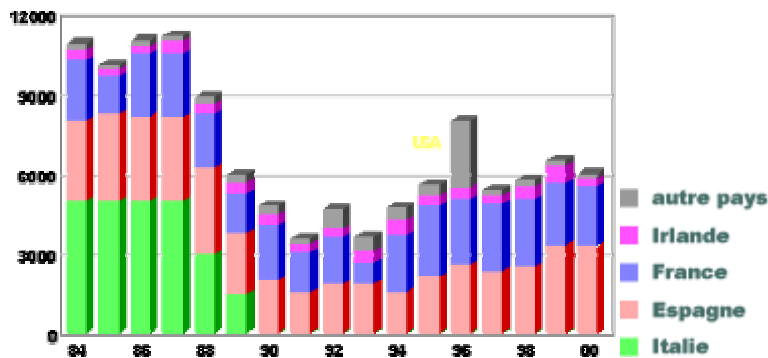
France, have resulted in a 20% decrease in employment in the oyster industry and a reduction of \$240 million in turnover. *Bonamia ostreae* was initially diagnosed in France in Tudy Island, Brittany in June 1979, but by 1980 the parasite had been found in all oyster growing areas in the region. It is thought to have been originally introduced into the region with a consignment of spat imported from California (Pichot *et al.*, 1980).

In recent years the production of *O. edulis* has been maintained within Brittany, but it is at a lower level than that prior to the introduction of the *B. ostreae*. Production has been maintained by having fewer people working in the industry and by modified husbandry techniques. Although 99.4% of spat is collected from natural settlement on limed tiles and on mussel shell, there is a move towards using hatchery seed, with 2.2 million spat supplied from hatcheries in 2000. As the seed oysters show a low prevalence of infection with *B. ostreae* and infection levels correlate with age, the plan to re-introduce the flat oyster, after massive eradication of the adults, entails low-density culture in deep, open water (in the Cancale area). This technique allows the culture of several hundred tonnes of flat oysters on a three year cycle.

The two main areas of spat production are Brest and Quiberon, and the main growth areas are Quiberon and Cancale. All of the Brest spat production and one third of the Quiberon spat are moved to Cancale when 10 months old, for on-growing in deeper waters. Over 83% of adults are moved from the farms to one of four marketing areas prior to sale: Cancale, Belon, Golfe du Morbihan and Arcachon. During the period 1980-2004 over 41,000 oysters were sampled and screened for *B. ostreae*, mainly by screening of heart imprints, and less frequently by screening of tissue sections. Infected individuals were observed every year over that time period, with peaks in infection being observed in 1994/1995 at a frequency of 30 to 35%, and to a lesser extent in 2001/2002 with a frequency of approximately 20%. Generally each year a higher frequency was observed in adults, but some infection was observed in spat and juveniles. Results indicate that some tolerance to the parasite has evolved in the oysters, resulting in fewer outbreaks, and allowing a certain level of production to continue in Brittany.



**Figure 11.** Production of flat oysters in tonnes in France from 1964 to 1990 ([http://www.ifremer.fr/aquaculture/en/molluscs/flat\\_oysters.htm](http://www.ifremer.fr/aquaculture/en/molluscs/flat_oysters.htm))



**Figure 12.** Flat oyster production throughout Europe between 1984 and 2000.

[http://www.ifremer.fr/aquaculture/en/molluscs/flat\\_oysters.htm](http://www.ifremer.fr/aquaculture/en/molluscs/flat_oysters.htm)

It appears that the industry in Brittany has been able to maintain itself, but at a much lower level than that prior to the introduction of *B. ostreae*. More input occurs during the production of spat and particular areas are chosen for on growing in deeper waters. Over the years, along with decreased oyster movements resulting in less stress for the oysters, it appears that some degree of tolerance to the parasite has developed in the oysters. A smaller number of people are involved in the industry, but this possibly allows more control over each part of the process and more monitoring of stocks.

## CHAPTER 5. METHODS OF CONTROL OF THE PARASITE / REDUCTION OF IMPACT OF DISEASE

Since eradication does not seem to be possible, once bonamiosis is present in an area, the only option is to control the disease as effectively as possible. Control of stocks, to prevent spread to new areas, is particularly important. Once *B. ostreae* has been introduced into an area, changes to husbandry techniques can, in some instances, reduce the impact of the parasite. Approaches, such as reducing handling, decreasing stocking density, minimising the impact of dredging and on growing in deeper waters, have all been thought to reduce intensity of infection, prevalence of infection and/or mortalities (Le Bec *et al.*, 1991; Robert *et al.*, 1991; Montes *et al.*, 1991; Lama and Montes, 1993).

### Resistance

A number of studies have been conducted to investigate the ability of oysters, exposed to *B. ostreae*, to develop resistance to this parasite. These studies have been both laboratory- and field-based and both small and large scale. Initially, in laboratory trials, a protocol was developed to isolate parasites and allow the 50% infectious dose to be determined. This protocol was then used to infect oysters and determine if any were showing evidence of resistance to the disease (Hervio *et al.*, 1995). Preliminary results from that study indicated that F1 progeny of oysters, that survived previous outbreaks of bonamiosis, were more resistant to *B. ostreae* than the parental stock (Hervio *et al.*, 1995).

Field trials were undertaken in Ireland in the early and the mid 1990s, to investigate the relative susceptibility of several Irish stocks to *B. ostreae*. These included Cork Harbour oysters, where bonamiosis is present, and Belmullet, Tralee and Lough Foyle oysters, all of which were disease free at the time of the trials (Culloty, 1992 and Culloty *et al.*, 2001). In initial short field trials, which were held in Cork Harbour in 1990/1991, there was no evidence of differential survival of any Irish populations. However, these field trials were very short; generally of 3-6 months duration (Culloty, 1992). In subsequent field trials in 1996/1997 some differences in susceptibility were observed. Over a period of 18-24 months, Rossmore oysters, which had been exposed to the parasite in Cork Harbour for over ten years, performed better than other Irish stocks in terms of survival and prevalence of infection, indicating that some level of resistance had built up in this stock (Culloty *et al.*, 2001). A major EU funded study, comparing relative susceptibility to bonamiosis of European stocks, also indicated that naïve oysters (previously unexposed) performed less well than previously exposed stocks. In that study, oysters from Rossmore and Lake Grevelingen which had previously been exposed to *B. ostreae*, performed better than oyster populations from Tralee, Lough Foyle, Mull and Loch Kishorn in Scotland and a population from Finistere, Brittany, when relayed in *B. ostreae*- endemic areas (Culloty *et al.*, 2004)

Some work on oyster breeding programs, to produce increased resistance to Bonamiosis, has also taken place in France, with some degree of success (Martin *et al.*, 1993; Boudry *et al.*, 1996; Baud *et al.*, 1997; Naciri-Graven *et al.*, 1998 & 1999) but issues of inbreeding and bottlenecks remain to be addressed (Launey *et al.*, 2001; Culloty *et al.*, 2004).

## CHAPTER 6. EU CONTROLS AND MONITORING

It has not been possible to eradicate bonamiosis from any area to date (Van Banning 1985 and 1987), once the parasite has been introduced. As a result, the emphasis of disease management is on prevention and control.

Prevention approaches are designed to ensure that *B. ostreae* is not transferred into new areas. Within the E.U., countries are required to monitor regularly for a number of diseases, which are notifiable: i.e. their occurrence must be notified to the EU (Anon, 1994). The main legislation dealing with movements of molluscs include:

- **Council Directive 91/67/EEC** of 28<sup>th</sup> January 1991 concerning the animal health conditions governing the placing on the market of aquaculture animals and products,
- **Council Directive 95/70/EC** of 22<sup>nd</sup> December 1995 introducing minimum Community measures for the control of certain diseases affecting bivalve molluscs,
- **2003/390/EC: Commission Decision** of 23<sup>rd</sup> May 2003 which established special conditions for placing on the market of aquaculture animals species considered not susceptible to certain diseases
- **2002/300/EC: Commission Decision** of 18<sup>th</sup> April 2002 established the list of approved zones with regard to *B. ostreae* and/or *M. refringens*.

**The current Directive (2006/88/EC)** was published in October 2006, and will replace previous Directives relating to movements of fish and shellfish within the EU, by August 2008. In relation to animal health requirements for placing animals and products on the market, the 2006 Directive deals with general requirements for transport of animals, e.g. ensuring necessary disease prevention measures are applied during transport and at the place of destination, and that all movements of animals are accompanied by health certification. For farming and restocking, animals must be clinically healthy, and must not come from an area with unresolved mortalities, and if required, known vector species and wild aquatic animals should be held in quarantine before relaying to a disease free area. The Directive also includes the measures that are required in relation to notification, control and epizootic investigations that should take place, if unusual mortalities occur, or if a possible pathogen is present. Movements of oysters are permitted only between zones in such a way that *Bonamia* cannot be transferred into a new area: viz. from bonamiosis-free to bonamiosis-free, from free to infected, or infected to infected areas.

### Sampling methods

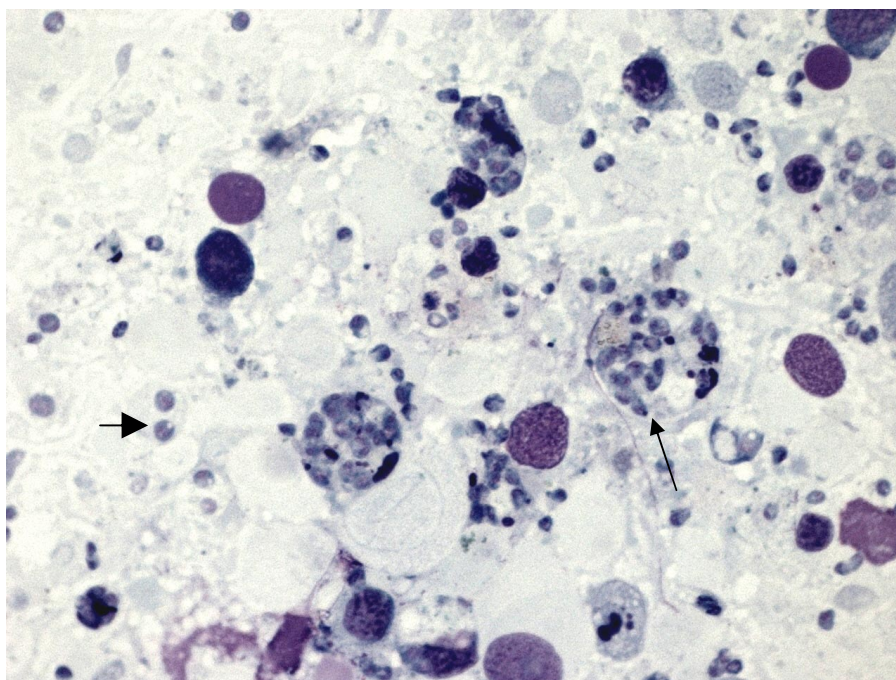
Sampling and diagnostic methods for screening shellfish are prescribed (Anon, 1994; OIE, 2006). **2002/878/EC: Commission Decision** of 6<sup>th</sup> November 2002 established the sampling plans and diagnostic methods for the detection and confirmation of the presence of the diseases Bonamiosis (*B. ostreae*) and Marteiliiosis (*M. refringens*). In the case of bonamiosis, samples of 30 oysters from each oyster bed are examined by heart smears and histology (OIE, 2006), twice a year. However, it is recognised that the disease may be not detectable by these methods during the first five months of infection. Results are notified to the E.U. These results provide information on the areas in which bonamiosis occurs, and those which are free of the disease.

Because the complete life-cycle of *B. ostreae* is uncertain, the possibility, that there may be vectors or intermediate hosts of the parasite *B. ostreae*, has not been ruled out. Transmission studies have examined the possible role of other bivalves, grown in the same areas as *B. ostreae* infected oysters, such as *Crassostrea gigas*, *Mytilus edulis*, *Ruditapes philippinarum*, *R. decussates* (Culloty *et al.*, 1999) and *M. galloprovincialis* (Figueras and Robledo, 1994) in bonamiosis, and concluded that these bivalves did not appear to act as either vectors or as intermediate hosts for the parasite. An earlier study by Renault *et al.* (1995), which involved

inoculation of *C. gigas* with purified *B. ostreae*, had not resulted in bonamiosis. Thus movements of these species are not affected by movement controls related to bonamiosis. However, these conclusions are based on diagnostic methods which recognise a conventional form of *B. ostreae*. The authors pointed out that the presence of a currently unrecognised form of the parasite could not be ruled out. At the time of that work only microscopic methods were available to detect *B. ostreae*. Since 2000, molecular based methods have become available which can be applied to similar studies, and a number are under way e.g. Marine Institute Project ST/05/25 in University College Cork,, investigating the molluscs, *C. gigas*, *Pecten maximus* and the abalone *Haliotis discus Hanoi*, as possible vectors or intermediate hosts of *B. ostreae* (2005-2007).

## CHAPTER 7. THE BIOLOGY OF THE PARASITE

*Bonamia ostreae*, which is also referred to as a microcell because of its small size (2 – 5 microns in diameter), is assigned to the taxonomic group, Haplosporidia, by the presence of haplosporosomes and on the basis of molecular analysis of the SSU rRNA gene (Carnegie *et al.*, 2000; Cochenec *et al.*, 2000; Cochenec-Laureau *et al.*, 2003a; Reece *et al.*, 2004), even though a spore stage has never been observed. The more recent discovery of *B. perspora*, a sister species to *B. ostreae*, with its clear capacity to produce spores, further supports the inclusion of *B. ostreae* in the Haplosporidia. (Carnegie *et al.*, 2006).

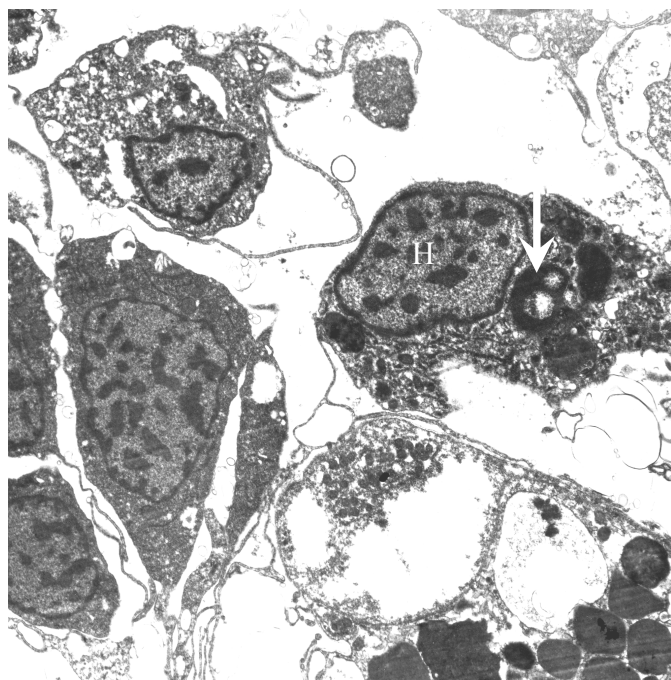


**Figure 13.** Heart smear showing extracellular (➔) and intracellular (➔) *Bonamia ostreae* cells within oyster haemocytes (blood cells)

*Bonamia ostreae* parasitizes the blood cells, or haemocytes, and the cells of the gill of the oyster. It may also be seen free in the tissues. The early stages of the infection are often accompanied by an infiltration of haemocytes into the tissues of the gill and mantle and around the gut (Balouet *et al.*, 1983; Poder *et al.*, 1983; Cochenec-Laureau *et al.*, 2003b). Many of these haemocytes contain *B. ostreae*, which multiply within them, and then lyse or rupture, releasing the parasites (Balouet *et al.*, 1983). The parasite multiplies by binary fission. Ten or more parasites can be observed within an infected haemocyte (Poder *et al.*, 1983). More rarely, during the terminal stages of the disease, a larger plasmodial form occurs, which contain 3 to 5 nuclei (Brehelin *et al.*, 1982). Infected oysters may appear normal, but heavy infections are associated with loss of condition and the presence of lesions in the connective tissues of gills, mantle and digestive gland. The disease may be lethal.

Descriptions of *Bonamia* sp. have been based on both light and electron microscopy studies (Hine, 1991b). Hine (1991b) in an ultrastructural study of *Bonamia* sp in *Tiostrea chilensis* described five stages of development of the parasite. Stage 1 is described as a dense form and was distinguished by its small size, few haplosporosomes and dense ribosomes, except around the cell periphery; Stage 3 an intermediate dense form with an irregular cell and nucleus shape and a golgi detached from the nucleus; Stage 5 is a plasmodial stage with an irregular shaped cell and nucleus, but also containing multivesicular bodies and large arrays of smooth endoplasmic reticulum. Stages 2 and 4 are transitional stages. Based on seasonal observations,

a tentative life cycle was proposed, with an incubation phase (September to November – Spring), a proliferation phase (December to May (Summer, Autumn) and a plasmodial phase (June to August – Winter) (Hine, 1991a). The relevance of these observations to the life cycle of *B. ostreae* in *O. edulis* in the Northern Hemisphere is unclear.



**Figure 14.** Electron micrograph of oyster hemocyte (H) and intracellular *B. ostreae* (→).

All ages of *O. edulis* are susceptible to infection. Two years of age seems to be a critical age in European oysters (Robert *et al.*, 1991 and Culloty and Mulcahy, 1996), nevertheless both 0+ and 1+ *O. edulis* can develop a high prevalence and intensity of infection (Lynch *et al.*, 2005). However, size of the animal may also be significant in terms of susceptibility (Cáceres-Martínez *et al.*, 1995). Male and female oysters appear to be equally affected (Culloty and Mulcahy, 1996).

## CHAPTER 8. LIFE CYCLE OF *B. OSTREAE*

Transmission of *B. ostreae* can occur directly from oyster to oyster, as has been previously shown in the laboratory (Culloty *et al.*, 1999), which would explain the rapid spread of infection on oyster beds, and suggest a direct life cycle. The disease can be experimentally transmitted, either by cohabitation of infected oysters with naïve oysters, or by inoculation of purified *B. ostreae* suspensions into uninfected oysters (Elston *et al.*, 1987; Miahle *et al.*, 1988a; Hervio *et al.*, 1995; Culloty *et al.*, 1999).

Infection develops after an apparent 'latent' period, which varies from four weeks to several months, before the parasite can be detected in the tissues (Poder *et al.*, 1983; Grizel *et al.*, 1988; Bucke, 1988; Montes, 1991; Culloty and Mulcahy, 1996, Culloty *et al.*, 2001). The events during the latent period of bonamiosis are unknown. Neither the parasite, nor another form of the parasite has been detected by histological techniques. Molecular techniques may provide more information on the disease mechanisms occurring during this period of the infection.

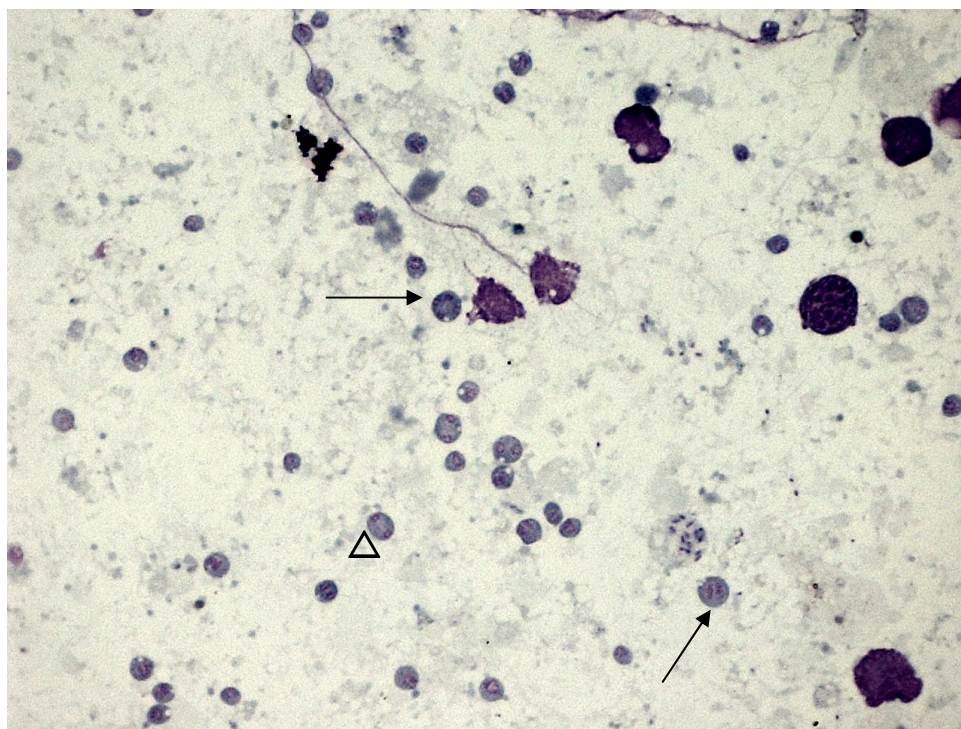
Transmission occurs throughout the year (Culloty and Mulcahy, 1996), although the prevalence of infection in oysters tends to increase during summer to autumn (Grizel *et al.*, 1988; Culloty and Mulcahy, 1996).

It appears that the parasite initially enters the oyster possibly during filtration of seawater or respiration (Bucke, 1988, Montes *et al.*, 1994). After passing through the gill epithelia (Montes *et al.*, 1994) the parasite is phagocytosed by the haemocytes, where it multiplies by binary fission, the haemocyte ruptures, and the parasites are free to infect other blood cells. Both intra and extra-cellular parasites are released from the oyster with pseudo faeces, and/or when the oyster dies, the released parasites are free in the seawater for an unknown period of time, to infect other oysters. Plasmodial stages develop sporadically, but it is unclear what factors might contribute to their development. The stages of the parasite that are routinely observed are uni- or bi-nuclear stages, either intra- or extra cellular, and, more sporadically, a multinucleated plasmodial stage. The uni-nucleated stage can appear either 'dense', where the nucleus is small and condensed, or 'light', where the nucleus and cytoplasm stain less intensely.

There are reasons to suspect that the parasite life cycle may involve an intermediate host or vector, even though direct transmission of *B. ostreae* is possible. For example, a spore stage is characteristic of the Haplosporidia, the group to which *B. ostreae* belongs. Though a spore stage has not been observed in *B. ostreae*, recently a spore stage has been described in the related *Bonamia perspora* which infects *Ostreola equestris* (Carnegie *et al.*, 2006). Also, Van Banning (1990) demonstrated that, when an oyster bed was cleared and left fallow for several years the naïve oysters, reintroduced into the area, quickly developed infection, supporting the idea that a reservoir of infection had persisted, perhaps in an intermediate host or vector, or in a spore form.

Another aspect of the life cycle and the progression of infection that remains unanswered is the 'latent period' i.e. the weeks following initial infection of the oyster. The form of the parasite, as it exists during this period, has not been observed to date. Knowledge of the complete life cycle of *B. ostreae* could inform future management strategies and control mechanisms, designed to minimise the influence of this parasite.





**Figure 15.** Binucleate ( $\blacktriangleright$ ) and plasmodial ( $\rightarrow$ ) stages of *B. ostreae*

#### ***B. ostreae* in Benthic Organisms?**

Until recently the only methods available to study the life cycle stages have been the use of light microscopic methods, the sensitivity of which are limited. The development of molecular techniques has made newer approaches possible. A recent EU CRAFT study (Q5CR 2002-72238) based in Cork harbour, allowed the invertebrate community in that area, where *B. ostreae* is endemic, to be identified and monitored over a 15 month period (Lynch *et al.*, 2006). Species, which have life cycles, compatible with a role as a possible intermediate host or vector for *B. ostreae*, were identified. Eight benthic invertebrates - *Sabella pavonina*, *Carcinus maenas*, *Ophiothrix fragilis*, *Nephtys hombergii*, *Actinia equina*, *A. aspersa*, *Axinella dissimilis* and *T. benedii*, as well as grouped zooplankton samples, were found to be positive when screened for *B. ostreae* (Lynch *et al.*, 2007). Subsequent laboratory trials, which involved holding a sub-sample of invertebrates: *Ophiothrix fragilis*, *Nephtys hombergii* and *Actinia equina* with naïve oysters, demonstrated that a small number of oysters developed infection when held with the brittlestars (Lynch *et al.*, 2007). However, the basis of how this transmission occurred is unknown. A positive result obtained from these invertebrates could be indicative of parasitism, or merely portage or ingestion of *B. ostreae* by the brittle star.



**Figure 16.** The brittlestar *Ophiothrix fragilis*

## CHAPTER 9. THE IMMUNE SYSTEM IN OYSTERS – THE HOST RESPONSE TO INFECTION

Few studies have been carried out, and surprisingly little information is available, on the immune system of the flat oyster *Ostrea edulis* (Hervio *et al.*, 1991; Morvan *et al.*, 1994; Cronin *et al.*, 2001; Culloty *et al.*, 2002). The general understanding is that the oyster's immune response to infection, i.e. its ability to mediate and fight infection or disease, is carried out by the blood. This consists of haemocytes or blood cells, which deal with the cellular response, and the non-cellular component, which deals with the humoral response. Three different morphological haemocyte types, that could be associated with different functions, have been described in *O. edulis*: granulocytes with granulated cytoplasm, and two agranular cell types, small and large hyalinocytes, which differ in the relative amount of cytoplasm (Cheng, 1981 and Auffret 1989). In bonamiosis, both granular and agranular blood cells types are infected by the parasite. In a study of the effect of *B. ostreae* on the haemocytes, Cochenec-Laureau *et al.* (2003b) found no difference in total circulating haemocyte counts, when comparing infected and uninfected oysters. The proportion of agranular cells increased in infected oysters, possibly as a result of granular cells being destroyed or being degranulated, as they fought infection, suggesting that the agranular cells might play a role in parasite survival and/or development.

Phagocytosis of foreign particles, including pathogens, by haemocytes is the main cellular immune response of bivalve molluscs (Cheng, 1981, Carballal *et al.*, 1997, López *et al.*, 1997). Once the pathogens have been internalised through phagocytosis, the haemocytes employ various mechanisms to kill and destroy them. One of these mechanisms comprises a metabolic pathway, known as the respiratory burst, in which oxygen is partially reduced to a number of powerful microbicidal metabolites, known as reactive oxygen intermediates (ROIs). These include superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), singlet oxygen ( $^1O_2$ ) and hydroxyl radical (OH) (Arumugam *et al.*, 2000a and b). Other ROIs, include nitric oxide (NO), which acts as a macrophage bactericidal agent (Hibbs *et al.*, 1988), and peroxyxynitrite, which can damage subcellular organelles, membranes and enzymes of the pathogen through its action on protein, lipids and DNA.

The haemocytes of *O. edulis* do phagocytose *B. ostreae*. However, the parasite, once within the phagocytic haemocyte, appears to have some counter mechanism, presently unknown, which can turn off the haemocyte's metabolic destructive capacity, so that the parasite survives within the haemocyte. Within this protected environment *B. ostreae* feeds and multiplies. Recent work on the interaction between *Perkinsus marinus* and its host *C. virginica* at the cellular level indicates that the parasite is able to suppress the production of ROIs (Schott *et al.*, 2003). This work could be used as a model system to investigate host/parasite interaction between *B. ostreae/O. edulis*. The cellular immune response of *C. gigas*, (which is not susceptible to bonamiosis), has been compared to that of *O. edulis* (which is susceptible). While *B. ostreae* is picked up and phagocytosed by both oyster species, *B. ostreae* is destroyed within the *C. gigas* phagocytes only (Chagot *et al.*, 1992; Mourtan *et al.*, 1992; Xue and Renault, 2000). Information on the basis of the response of the two species to *B. ostreae* could provide valuable information on strategies for counteracting the effect of the parasite.

There are a variety of non-cellular reactions to parasites and pathogens in bivalve molluscs including antimicrobial compounds, proteins with protease inhibitory activity e.g. in *C. virginica* infected with *Perkinsus marinus* (Faisal *et al.*, 1998), phenoloxidase which participates in the encapsulation and melanisation of foreign bodies (Söderhäll, 1982) and heat shock proteins (HSPs), whose synthesis increases in response to high temperatures and which may protect against other physical, chemical and biological stresses in the environment such as disease (Clegg *et al.*, 1998). The role, played by non-cellular components in countering the development of bonamiosis, has not been elucidated.

## CHAPTER 10. DIAGNOSIS OF *B. OSTREAE*

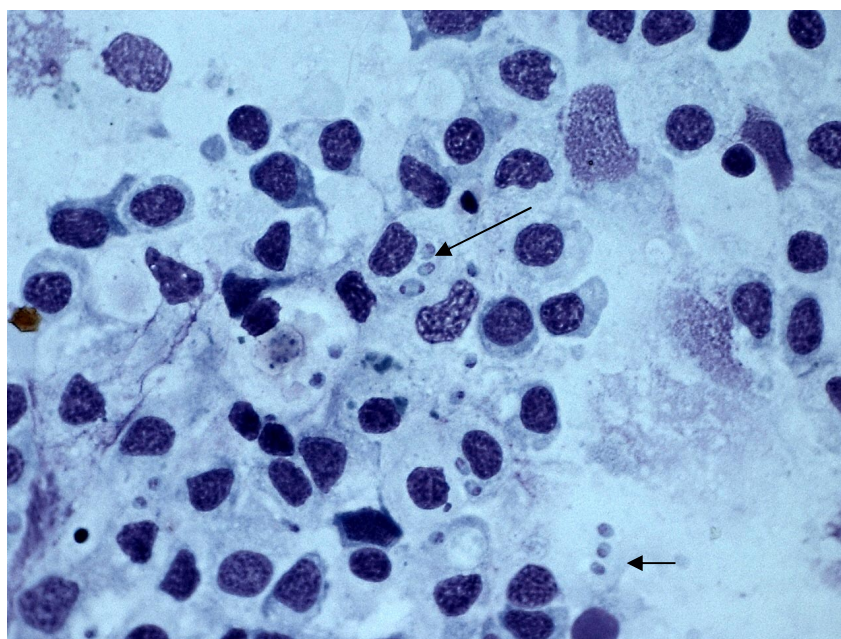
A range of diagnostic techniques based on light and electron microscopy, and molecular and immunological techniques have been developed to detect the presence of this parasite.

### Light microscopy

The techniques include screening of ventricular heart smears and tissue sections. The ventricular heart smear technique involves removal of the ventricle of the oyster heart, and dabbing the tissue onto a clean glass slide. The resulting imprint of blood cells is air dried, fixed in methanol and stained in a commercially available reagent (Hemacolor 2 and 3, Merck). The blood cells are screened for the presence of intra and extracellular parasites. This technique may also be used to screen previously frozen oysters, if they are handled carefully (Rogan *et al.*, 1991). The advantage of this technique is its speed: slides can be immediately examined microscopically, and the cost is relatively low.

### Histology

This involves fixation of the oyster tissue and subsequent sectioning (cutting thin slices) of the tissue, followed by staining. The stained sections are then examined under the microscope. This preparation process takes approximately one week. Only a small fraction of the oyster tissues is screened. However, advantages of histology are that infections of *B. ostreae* are often accompanied by foci and infiltration of infected hemocytes into the tissues, which would not be observed with other techniques (Bucke and Feist, 1985). Also the general health of the animal can be determined and other infections detected.



**Figure 17.** Heart smear with extracellular *B. ostreae* (←) cells

### PCR techniques

Three Polymerase Chain Reaction (PCR) techniques, based on different primer sets, have been developed recently (Carnegie *et al.*, 2000; Cochenec *et al.*, 2000; Marty *et al.*, 2006) allowing detection of the parasite's DNA within the oyster tissues. A recently developed primer pair (Marty *et al.*, 2006) produces a smaller DNA product, which may increase sensitivity over the earlier primer pairs. A Realtime PCR assay for detecting *B. ostreae* has been developed, based on the analysis of fluorescence in 96 well plates, allowing for rapid throughput and quantification of the DNA product (Marty *et al.*, 2006). A Realtime TAQMAN PCR assay has also been developed to detect *Bonamia* species in infected oyster tissue (Corbeil *et al.*, 2006).



This method can be optimised to quantify the parasite load in samples. This assay detected isolates from Europe, Canada, Australia, New Zealand, Chile and the USA and therefore demonstrated genus specificity. In studies to date, it appears that PCR is more sensitive than light microscopy methods, and it allows for detection of light infections that might otherwise go undetected (Carnegie *et al.*, 2000; Lynch *et al.*, 2005; Balseiro *et al.*, 2006; Corbeil *et al.*, 2006).



**Figure 18.** Gel showing results of PCR screening of oyster tissue for *B. ostreae*

An *in situ* hybridization (ISH) assay has also been developed for screening for *B. ostreae* (Cochennec *et al.*, 2000; Carnegie *et al.*, 2003). ISH allows amplification and detection of the parasite DNA within a section of the oyster tissues, allowing the location of the parasite to be visualised. This technique has applications and advantages. For example, for study of the life cycle of the parasite, it allows the possibility of tracking the infection during the ‘latent period’, and elucidating the role of other organisms in the life cycle, found positive for *B. ostreae* by PCR.



**Figure 19.** *In situ* Hybridisation (ISH) of tissue section of infected *O. edulis* (blue stained cells are *B. ostreae* orange staining – connective tissue)

### Evaluation of Methods.

The OIE recommended methods available and used for diagnosis of *B. ostreae* are heart smears, histology, PCR, PCR–RFLP (Restriction Fragment Length Polymorphism) and *in situ* hybridisation, with different methods being prioritised depending on the samples being screened (OIE, 2006). For routine surveillance in known host and geographical ranges, tissue imprints are the recommended method for screening, with PCR and histology being standard methods with good sensitivity and specificity. ISH may have some applications, but PCR-RFLP and TEM are not recommended under these circumstances. For surveillance outside the host and geographical range, histopathology is the recommended method. For a presumptive diagnosis, tissue imprints, histopathology and PCR are recommended, with histology being the preferred method outside the host and geographical range. For confirmatory diagnosis PCR-RFLP and TEM are the recommended techniques.

A number of other approaches have been taken over the years, to try to develop sensitive and rapid techniques for screening for *B. ostreae*. Immunology based techniques have been developed, with the production of monoclonal antibodies against *B. ostreae* (Rogier *et al.*, 1991). An immunofluorescent technique was developed by Mialhe *et al.* (1988b); six cloned hybridomas secreting monoclonal antibodies against *B. ostreae* were selected. However differences in detection rates were observed when the immunofluorescent technique was compared with histological based techniques (Boulo *et al.*, 1989). The immunofluorescent technique also gave unclear results when tested extensively on oysters from Maine, USA (Zabaleta and Barber, 1996). Subsequently an ELISA based diagnostic kit was developed which was reported to have a 90% agreement when compared to light microscopic examination for the parasite (Cochennec *et al.*, 1992). However this was later abandoned.

Each of the techniques developed to date has advantages and disadvantages. Heart smears are rapid and relatively inexpensive though they require a trained observer to screen the slides. In a study on oysters in Cork Harbour, however, O' Neill *et al.* (1998) found that when use of heart smears and haemolymph smears (a drop of blood) were compared, a slightly higher prevalence of infection was detected by examination of the haemolymph smears. Gill smears are also used for screening of blood cells and do not appear to differ from heart smears in their sensitivity (da Silva and Villalba, 2004). A sufficiently large sample size is also critical when oysters are being screened for *B. ostreae* using these techniques, as recently-infected animals may not be diagnosed, if infection is at the latent period stage (Culloty *et al.*, 2003). Da Silva and Villalba (2004) also raised concerns about the sensitivity of light microscope based techniques.

Molecular based techniques appear to be more sensitive than traditional methods for diagnosing infection. The PCR assay proved to be more sensitive, more specific and less ambiguous than standard histological and cytological (tissue imprint) techniques, when initially evaluated by Carnegie *et al.* (2000). Recent studies screening oysters for *B. ostreae*, using both molecular and light microscopy based techniques, demonstrated that screening by PCR yielded a higher prevalence of infection than the other techniques (Lynch *et al.*, 2005; Balseiro *et al.*, 2006). Molecular techniques may also play a role in more sensitive screening of oyster larvae and spat, when histopathology may be inadequate (Lynch *et al.*, 2005 and Marty *et al.*, 2006). Diggles *et al.* (2003) in a study of diagnostic techniques for *B. exitiosa* found that ISH was more sensitive than either PCR or light microscopy, and was useful for screening small numbers of oysters, where high sensitivity is required. Furthermore ISH allowed detection of low intensity infection, undiagnosed by other methods. It appears that no one method on its own is entirely satisfactory, and a combination of techniques should be employed when screening to give a true reflection of the prevalence of infection by *B. ostreae*.

The evaluation of diagnostics for *B. ostreae*, and the selection of suitable techniques for screening, are important on a number of levels. Ease, speed and cost of the technique must be balanced with reliability and sensitivity. Using light microscope techniques as the only diagnostic methods in the control of the movements of stocks between regions may result in infections going undetected, and the introduction of an infected stock into a zone free of *B. ostreae*. Screening of heart smears or tissue sections should be backed up by use of PCR, to assess the true level of infection. PCR is a useful tool when assessing both the prevalence of infection and spread of infection within a bed, as it may give a more realistic picture of infection levels than light microscopy based techniques. Similarly, in the weeks following initial infection it is often difficult to detect the parasite in smears or tissues. PCR again may be required to diagnose infections in these instances. However, visualising the parasite and/or sequencing the product is also required to confirm that the DNA detected by the PCR is in fact that of *B. ostreae*. ISH may now allow us to gain some insights into what is happening within the hosts' tissues during the latent period of infection, and determine if a particular tissue is being targeted initially, or if another stage of the parasite is present. It will allow progression of the disease to be monitored at this early stage of infection. Reproducibility of methods between laboratories is also a requirement, and one recent study (Balseiro *et al.*, 2006) found over 90% similarity between two laboratories screening for *B. ostreae* when using light microscopy and molecular techniques, - nevertheless leaving a margin of error in the results obtained between the laboratories.

## CHAPTER 11. RESEARCH ISSUES

### **Life cycle of *Bonamia ostreae*:**

#### *Ultrastructure of the parasite:*

Apart from some initial early work (Pichot *et al.*, 1980; Breheling *et al.*, 1982; Bonami *et al.*, 2005), the ultrastructure of the various stages of *B. ostreae* has not been studied, to establish the developmental sequence of the parasite in the oyster, and to determine if they are the same as those observed for *Bonamia* sp. in *Tiostrea chilensis* by Hine (1991b).

#### *Other possible stages and forms of the parasite:*

The physical form of the parasite during the latent period is unknown i.e during the initial stages of infection. Furthermore, it is not known whether there are additional stages of the parasite, which occur outside the oyster host. Nor is it known whether the parasite uses an intermediate host as a location for development, and if so, of the thousands of possible species, what the identity of the intermediate host might be (Lynch *et al.*, 2006 and 2007). Recently developed molecular techniques, such as PCR and *in situ* hybridisation provide tools, which may help to answer some of these questions.

#### *Is there an intermediate host in the life cycle?*

Initial studies to identify a possible intermediate host in bonamiosis, which necessarily involve a “needle-in-haystack” approaches, have given results which suggest that one of a number of species may be an intermediate host (Lynch *et al.*, 2006 and 2007). These results need to be followed up with more extensive PCR screening of invertebrates and zooplankton in the field, as well as larger scale targeted laboratory studies, involving trial disease transmission between potential intermediate hosts and naive oysters, using a range of methods including PCR and *in situ* hybridisation.

#### *Strain variation in *Bonamia*:*

While work is ongoing in Spain and elsewhere on genetic variation in oyster populations, no such work has been carried out on *B. ostreae*. This work needs to be undertaken to establish the variation in strains from different sources, and the relative virulence of the different strains. This is particularly relevant to the likely future movements of more resistant oysters, for restocking depleted bonamiosis-infected areas.

#### *Carriers: biotic and abiotic:*

Movements of *B. ostreae*-infected oysters from one location to another, and from one country to another have been at least partly responsible for the spread of bonamiosis. EU Regulations try to prevent future spread, by the control of movements of oysters between infected and non-infected areas. It is known that *O. edulis* has been accidentally included in consignments of other bivalve species, which were legally moved from other area to another, and so could transfer the parasite to new areas. What is not known is whether species of other commercial bivalves can act as carriers of bonamiosis, while not susceptible to the disease themselves. One investigation, carried out in 1999 (Culloty *et al.*, 1999), which used traditional methods of diagnosis, found no evidence that *C. gigas*, *M. edulis*, *M. galloprovincialis*, *Ruditapes philippinarum* or *R. decussatus* acted as carriers. However the authors cautioned, since the life cycle of the parasite is not fully known, that the possibility could not be ruled out, of the parasite in an undetected form being carried by these other bivalves. A further study (Marine Institute.S.T. 05/25) is under way at present, using molecular as well as light microscopy based techniques to try to answer this question in relation to a number of the bivalves and gastropods, which are commercially important in Ireland and Europe.

### **The immune system of the oyster, *Ostrea edulis***

There are two primary “players” in bonamiosis: the parasite, *B. ostreae* and the host oyster, *O. edulis*. For the parasite to successfully infect a potential host, it needs not only to successfully gain entry to the oyster, but also to overcome the immune defence system of the host. All living organisms have a sophisticated array of mechanisms, which have evolved to resist and overcome parasites and pathogens. It is surprising that so little is known about the immune mechanisms of *O. edulis*, considering the commercial significance of the species. In general it is known that bivalves have a range of cellular and humoral reactions, which target different components of the parasite’s devices. It is not known which particular cellular and humoral responses the flat oyster can mount against *B. ostreae*. Investigations into this question are at a relatively primitive stage, but they are at least as important as gaining an understanding of the biology and life cycle of the *B. ostreae* parasite, if the bonamiosis is to be successfully controlled and managed. A greater understanding of the interaction of other parasites and their hosts is available e.g. *Perkinsus marinus* and *C. virginica*, and these systems could be used as models for the study of *B. ostreae/O. edulis* interactions. The recent development of methods, such as suppression subtractive hybridisation and microarrays, could allow investigation into the genes encoding for these parameters, and allow investigation into the expression of the genes, when infection is developing.

While there is evidence that oyster stocks, bred from infected oysters, develop a degree of resistance to bonamiosis, there is as yet no understanding of the nature of this resistance, nor of the mechanisms involved, and these need to be investigated further.

### **Detection of *Bonamia ostreae*, and diagnostic methods**

The techniques available for detection of *B. ostreae* include microscopic examination of heart smears, histological examination of tissue sections, PCR, PCR-RFLP and *in situ* hybridisation. As outlined above, all of these methods have limitations. Thus all the efforts to manage bonamiosis, and EU and international regulations to prevent its spread, depend on a system of diagnosis that has limitations. Continual improvement of existing methods should be considered, not only by the generation of new primer sets with increased specificity for *B. ostreae* for improved PCR methods, but also by the development of additional, more reliable methods of detection. Currently the Cochenec primers available amplify all microcell haplosporidians, and the Carnegie primers amplify *B. ostreae* and *B. exitiosa*; thus neither assay is species specific. Use of some probes such as the Bo-Boas for ISH indicate that the method is not species specific as it also detects *Haplosporidium nelsoni* in *Crassostrea virginica* and *Bonamia exitiosa* in *Ostrea chilensis*. Also neither ISH nor PCR have been validated against histology (OIE, 2006).



## CHAPTER 12. RECOMMENDATIONS FOR THE HEALTH MANAGEMENT OF FLAT OYSTERS

### Objectives

- ◆ Prevent the spread of bonamiosis to disease free areas in Ireland
- ◆ Minimise the impact of *B. ostreae* in infected stocks
- ◆ Conservation and enhanced production of a native species *O. edulis*

#### 1. Disease Free Areas

The remaining flat oyster beds in areas which are bonamiosis-free, should be safeguarded, and maintained as single-species areas. Cultivation and movements of other mollusc species into these areas should be avoided, thus minimising the danger of transfers of *B. ostreae* or other pathogens. Currently, this is important while the issue of the possible role of vectors in the transfer of *B. ostreae* is being resolved. Appropriate biosecurity plans should be developed in these areas. Boats and equipment should be used in one area only, and not be moved between areas, because pathogens including *B. ostreae* could potentially be carried and passively transferred by them between areas.

This precautionary approach is advisable until such time as the full life-cycle and transmission of *B. ostreae* are understood, and until validation of all current diagnostics for bonamiosis have been carried out, and the limitations of all currently available techniques have been determined.

#### 2. Irish native oyster growing regions:

For all flat-oyster growing sites, whether *B. ostreae* is present or not, growing conditions should provide optimal water quality and optimal oyster densities. Oysters growing in deeper waters (in excess of 2 m), in areas where the parasite is endemic, appear to survive better than those in shallow waters. Stocks should be managed and local beds restored. This is a long-term process, which can be expected to take 25 years or more (Laing *et al.*, 2005). The management plans should involve all stockholders for the area. Movements of other shellfish species in and out should be minimised or, better still avoided. Scientific advice and stock surveys should be used in setting annual fishing targets, to avoid the risk of over-fishing.

#### 3. Stock enhancement

Ideally, *O. edulis* stock development in all areas should concentrate on building up self-sustaining stock. Otherwise, the stock should be supplemented by spat produced from the local oysters, rather than by the transfer of spat from other sources, particularly those outside Ireland, because introduced oyster strains may be less adapted to the local conditions, and therefore more vulnerable to diseases including bonamiosis (da Silva *et al.*, 2005). In areas where bonamiosis is already present, breeding from surviving oysters could gradually build up *B. ostreae*-resistant stocks (Culloty *et al.*, 2004). Long-term breeding of oysters from infected stocks should be continued and expanded, to further build the development of stocks which are more resistant to bonamiosis.

#### 4. Conservation and genetic diversity.

*Ostrea edulis* stocks should be managed and local beds restored. In order to conserve a native species, a survey should be undertaken of the genetic diversity around the coast. Interested stakeholders such as the oyster industry, Marine Institute, Department of Marine and Natural Resources and National Parks and Wildlife should consider the implementation of a conservation plan for this species.

## **5. Monitoring and Diagnosis**

Oyster fishermen and cooperatives, along with state agencies and Sea Fisheries officers, should take a proactive approach to ensure full coverage in the national monitoring program, in line with the EU Directives on fish and shellfish health.

Detection of *B. ostreae* depends on sampling host oysters. Because sampling is also a statistical exercise, results can never be 100% representative of the reality. These facts must be borne in mind when the sampling approach is being designed to detect the parasite, so that the sample size, most appropriate to the question being asked, as well as the appropriate methods, are used.

Inter-laboratory collaboration should be encouraged to allow ongoing validation of techniques for diagnosis and development of more sensitive techniques.

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