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Aspects of the biology of the parasite *Bonamia ostreae* with a view to gaining a greater understanding of how to alleviate its impact on the European flat oyster, *Ostrea edulis*.

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Thesis submitted to the National University of Ireland, Cork for the degree of Doctor of Philosophy

January 2014

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Declaration

I declare that this thesis has not been previously submitted as an exercise for a degree at the National University of Ireland or any other university and I further declare that the work embodied in it is my own, or else noted.

Grace Flannery

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Chapter 1

Introduction

Oyster production worldwide

According to the European Commission (2009), worldwide oyster production was nearly 4,604 million tons in 2004, 97% of that originating from aquaculture. The main oyster producer worldwide is China at 83%, followed by the Republic of Korea (6%) and Japan (4%). The Pacific oyster, *Crassostrea gigas,* represents over 80 % of the world's edible oyster production through aquaculture, having reached 728,552 tons in 2007. In the same year, 94,382 tons of the American oyster *Crassostrea virginica* were cultured and the European flat oyster *Ostrea edulis* production remained low at 6,357 tons (FAO 2008).

Oyster production in Europe now focuses mainly on *C. gigas*, which was introduced into Europe in the 1960s while *O. edulis* is currently produced in small volume. Total European oyster production for the year of 2005 was in the order of 132,000 tons and valued at nearly €300 million. The level of oyster production has decreased somewhat since the 1990s, in 1994 production was at 157,323 tons, decreasing to 130,199 in 2004 (European Commission 2009).

Oyster production in Europe is dominated by the French, who produce approximately 120,000 tons of *C. gigas* annually and a remaining 1,500 tons of *O. edulis* (Buestel et al. 2009). France has also become the largest consumer market for oysters in Europe (Buestel et al. 2009). Overall, French oyster production accounts for 2.5% of worldwide oyster production (FAO 2008). Ireland is the next largest producer with Pacific oyster production at 7,032 tons in 2007 and flat oysters at 382 tons (Marine Institute et al. 2010).

Distribution of *Ostrea edulis*

The European flat oyster *Ostrea edulis*' natural range extends from the coast of Norway, south to Morocco and into the Mediterranean basin as far as the Black Sea coast (FAO 2012). Wild populations are now extremely scarce around Europe with natural beds only surviving in several regions including the West Coast of Ireland and the Limfjord region of Denmark. *O. edulis* has been intentionally introduced in the USA, East Canada, Japan and eight other countries since the 1940s (Ruesink et al. 2005) but so far has only established itself in the eastern USA, where naturalised populations have been observed from Maine to Rhode Island on the north east Atlantic coast (FAO 2012).

Production of *Ostrea edulis*

Over the last forty years, European production of *Ostrea edulis* has shown a drastic decline from a peak output of nearly 30,000 tons in 1961 to 6,000 tons today, mainly due to the impact of two parasitic diseases, *Marteilia refringens* and *Bonamia ostreae* (Lallias et al. 2008). With on going infection with *B. ostreae* in *O. edulis* populations and a considerable shift to *Crassostrea gigas* farming, the flat oyster industry has been unable to recover to anything like the production figures achieved in the 1960's despite numerous efforts (Culloty & Mulcahy 2007, FAO 2012). Production has since remained low but stable, averaging 6,000 to 7,000 tons annually between the years 2000 and 2002 (FAO 2012). In 2002, 67% of flat oyster production was in Spain (4,565 tons) with 24% in France (1,600 tons). Ireland and the UK were the only other countries that produced more than 200 tons that same year (FAO 2012). The European flat oyster industry constituted less than 0.2% of the total global production of all farmed oyster species for 2002. However, the flat

oyster obtains a higher price on the market than other species, such as *C. gigas,* resulting in the value of farmed *O. edulis* production in 2002 being USD 24.3 million, thus its culture remains an important industry in the limited areas where it is reared (European Commission 2009, FAO 2012).

Ireland, as with the rest of Europe, suffered a dramatic decline in the natural flat oyster population, even before the introduction of any parasitic diseases, possibly due to overexploitation or a lack of management. Barry's (1980) study of national oyster beds throughout Ireland, stated that by the 1970s only four of the twenty-four native oyster fisheries in existence in 1900 still remained. Total market value for the flat oyster has been decreasing, in 2007 alone, it declined by €0.3 million to €1,630,000 (Marine Institute et al. 2010).

Habitat and Reproduction of *Ostrea edulis*

Ostrea edulis is prominently found in intertidal areas, in depths of up to 20m, but can also be found in estuarine areas (Lapegue et al. 2006). It can live up to 20 years and can tolerate salinities of up to 35 ‰ and as low as 15‰ (Lapegue et al. 2006). It is a sequential protandrous hermaphroditic oyster, changing sex after a reproductive cycle (Helm et al. 2006). The sexual inversion is influenced by temperature and food availability. Yonge (1960) reported that, in Scandinavia, the flat oyster changed sex once a year, while in Great Britain and in France each oyster could be male or female several times in the same summer (Heral & Deslous-Paoli 1991). Females are larviparous, so fertilisation occurs in the mantle cavity and brooding eggs are incubated to an advanced larval stage of 8 to 10 days before being released (Heral & Deslous-Paoli 1991, Helm et al. 2006, Lapegue et al. 2006).

Culturing of *Ostrea edulis*

Oyster seed is obtained by either collection of wild spat or from hatcheries (European Commission 2009). Wild collection most frequently occurs in areas with natural populations (most common in Ireland) while hatchery production occurs in areas with no natural supply (FAO 2012). In the hatchery production of flat oysters, artificial fertilization is considered fairly unsuccessful, in view of the extremely low survival rate obtained with larvae, as a brooding period is still required. Increasing seawater temperature and providing additional food brings about sexual maturation and reproduction of the species. Once the broodstock is ripe, the gonads are prepared for release of gametes, two methods of breeding may be used, the first being mass spawning, based on the maturation of a whole batch of oysters together, removing any control over cross-breeding and the genetic contribution of individuals to the new generation. The second method involves maturing only two oysters per tank and then collecting the resulting seed, permitting selective breeding (Helm et al. 2006, FAO 2012).

Most European flat oyster culture remains based upon the use of spat collectors to obtain wild juveniles. Modern spat collectors consist of either bags or tubular nets filled with mussel shell suspended under steel frames placed in waters of 3 to 6 metres in depth (Heral & Deslous-Paoli 1991, FAO 2012). In Cork harbour, on the south coast of Ireland, settlement of spat takes place on mussel cultch placed in land based spatting ponds.

After the seed collection, culture is carried out in two phases: pregrowing and maturing phase. The pregrowing stage involves the metamorphosis from seed to intermediate size, which takes one to two years. From there the oyster will enter the maturing phase where it will reach a marketable size. The amount of time it takes for

an oyster to reach marketable size varies from location to location due to temperature and food availability (Heral & Deslous-Paoli 1991) but in general is approximately 4 years. Currently the two main oyster culture methods used in Europe are off-bottom and on-bottom culture (FAO 2012). Off-bottom techniques include rack or raft cultures where oysters are grown in plastic baskets, oyster bags, floating trays, longlines or ropes suspended from metal trestles, rafts or racks (European Commission 2009, FAO 2012). The oysters are graded and thinned out as they grow (FAO 2012). On-bottom culture consists of sowing the oysters directly onto the intertidal sea-bed or in deep water of 5 to 10 metres in depth (Buestel et al. 2009), a common method deployed in Ireland.

Bottom cultured oysters are usually harvested in subtidal areas by boats with two steel dredges, operated by a hydraulic or pneumatic winch (FAO 2012). Harvests in intertidal areas can be done by hand (Heral & Deslous-Paoli 1991). Once oysters have reached their marketable size, approximately 60 g, farmers grade and place them in depuration systems for a period of 42 hours or more before marketing. Depuration usually involves placing oysters in a holding tank through which purified water flows. The water is purified with chlorine, ozone or ultraviolet light to remove pathogens (FAO 2012).

Pathogens and disease of oysters

Unusual mortalities, possibly from disease, have been observed in oyster populations worldwide since the 1920s (Orton 1923). The species most significantly impacted have been the American oyster, *Crassostrea virginica*, the European flat oyster, *Ostrea edulis*, and the Pacific oyster, *Crassostrea gigas*. Most of these mortalities have now been found to be the result of intracellular parasitic infections such as

Bonamia ostreae, Haplosporidium nelsoni, Perkinsus marinus, Marteilia refringens and *Mikrocytos mackini.*

In the 1960s, serious mortalities began occurring in Pacific oyster populations at Denman Island, British Columbia, Canada. Mortalities reached 35% in one *C. gigas* population (Quayle 1961, Bower 1988, Bower et al. 1994). The disease, now known as Denman Island disease, was discovered to be caused by *M. mackini*, a small, 2–4 μm, protistan parasite (Farley et al. 1988). The parasite itself is a protist of unknown taxonomic affiliation (Hine et al. 2001) and its mode of transmission remains unknown (Carnagie et al. 2003).

P. marinus is a pathogen of *C. virginica,* which causes Dermo disease. It occurs in populations along the North American coast from Maine to Florida to the Yucatán Peninsula of Mexico (Andrews 1996, Burreson & Ragone Calvo 1996, Ford & Tripp 1996, Gullian-Klanian et al. 2008). The disease is transmitted directly from oyster to oyster and has caused mass mortalities of *C. virginica* throughout its region (Burreson & Ragone Calvo 1996, Ford 1996, Ray 1996, Powell et al. 2008). Mortalities may range from 5 to 30% during the first year of an epizootic, and total from 60 to 80% by the end of the second year (Kern & Ford 2011). Activity of the disease is primarily regulated by temperature. Infectious stages of the parasite are present only in the warm months from May to October. Bushek & Allen (1996) found that some stocks of *C. virginica* are naturally resistant to *P. marinus*.

H. nelsoni or MSX (multi-nucleated sphere unknown), which also infects *C. virginica,* first appeared in Delaware Bay, USA in 1957 and Chesapeake Bay in 1959, where it caused mortalities throughout most of the year (Andrews 1982). It has been known to kill up to 95% of a population upon introduction (Haskin et al. 1966).

It co-exists alongside *Haplosporidium costalis,* which causes sporadic mortalities in *C. virginica* during May-June (Hine & Wesney 1992).

Transmission of the disease has now been confirmed to be caused by the involvement of water-borne infectious particles i.e. fish such as *Menidia menidia*, *Syngnathus fuscus* and *Fundulus sp.* and invertebrates such as *Crangon septemspinosa, Paleomonetes vulgaris* and *Neomysis americana* (Sunila et al. 2000). *C. gigas* are also susceptible to infection as *H. nelsoni* is thought to have been originally introduced to North America in the 1950's in a *C. gigas* consignment from Japan (Andrews 1984, Friedman 1996). Most recently *H. nelsoni* was detected for the first time in Pacific oysters in Ireland and also for the first time in a single *Ostrea edulis,* most possibly indicating an incidental infection, which may not have a significant impact on *O. edulis* (Lynch et al. 2013).

Though records of unusual mortality events in the native oyster extend back to the 1920's (Orton 1923), the main pathogens affecting *Ostrea edulis* are *B. ostreae* and *M. refringens. M. refringens* was first recorded in French waters in the late 1960s (Grizel et al. 1974), causing high mortality, over 90%, in European flat oyster populations grown in estuaries and since then it has been observed in several other oyster species including *Ostrea angasi* and *Ostrea chilensis* (Baud et al. 1997, Berthe et al. 1998). Cahour (1979) also reported *M. refringens* cells in the Pacific oyster. It is also known to infect the mussels *Mytilus edulis* (Auffret & Poder 1983) and *M. galloprovincialis* (Villalba et al. 1993). Both mussel species are not adversely affected by marteilioisis (Longshaw et al. 2001).

Experimental evidence showed that *M. refringens* cannot be spread by direct horizontal transmission, suggesting that intermediate or alternative hosts, or free-living stages, are essential in the life cycle of the parasite (Berthe et al. 1998).

Audemard et al. (2002) discovered that the copepod *Paracartia grani* acts as a host of *M. refringens*. A further six taxa of zooplankton were also found to be hosts of the disease (Carrasco et al. 2007).

Bonamia ostreae and the native oyster, *Ostrea edulis*

Bonamia ostreae is an intracellular haplosporidian pathogen of *Ostrea edulis,* found in Europe (Pichot et al. 1980, Balouet et al. 1983, Montes 1990, Hudson & Hill 1991, McArdle et al. 1991, van Banning 1991), on the Pacific coast of the USA (Elston et al. 1986, Friedman et al. 1989), and in Maine, USA (Barber & Davis 1994, Friedman & Perkins 1994). It is the causative agent of the disease bonamiosis, which has caused major mortalities among *O. edulis* throughout its distribution range (Lynch et al. 2007).

The parasite was first described in Brittany, France, by Pichot et al. (1979). The introduction and spread of *B. ostreae* in Europe is now believed to have occurred through multiple movements of infected oyster seed from California to France and Spain (Elston et al. 1986, Cigarría & Elston, 1997). *B. ostreae*, in conjunction with earlier mortalities caused by *Martelia refringens*, caused a drastic drop in the European production of *O. edulis* from 30,000 tons in 1961 to 6,000 tons today (Lallias et al. 2008).

Characteristics of the Haplosporidia

Bonamiosis is a disease of oysters, caused by a group of haplosporidian parasites of the genus *Bonamia*. The group Haplosporidia is composed of obligate histozoic and coelozoic parasites found in a variety of freshwater and marine invertebrates; with those in the genus *Bonamia* known specifically for oyster infection in euhaline to

polyhaline coastal environments (Burreson et al. 2000, Carnegie et al. 2006). Sprague (1979) noted that, historically, this group was treated as a last resort for an array of spore-forming parasites that have plasmodial cells in their life cycles and that were not easily classifiable elsewhere. The presence of uninucleate small (<5 μm) cells is also characteristic of microcell haplosporidians (Kroeck 2010). Cercozoa, the phylum to which *Bonamia* spp., belong are typically unicellular and uninucleate and undergo binary fission but some are multinucleate plasmodia or can temporarily form such plasmodia. Gross signs of infection such as cysts are commonly observed and associated with haplosporidian infections (Bass & Cavalier 2004).

Bonamia species worldwide

Currently there are five species acknowledged to belong to the genus *Bonamia*; *Bonamia ostreae* causing mortalities in *Ostrea edulis, Bonamia exitiosa,* which infects *Ostrea chilensis* from New Zealand and Chile, *Ostrea angasi* in Australia (Hine et al. 2001, Corbeil et al. 2006), and *O. edulis* in the Mediterranean and England (Carrasco et al. 2012, Longshaw et al. 2013), *Bonamia roughleyi*, formerly *Mikrocytos roughleyi* (Carnegie & Cochennec-Laureau 2004, Carrasco et al. 2012), which is the causative agent of Australian Winter Disease in *Saccostrea glomerata* (Farley et al. 1988), though recent research by Carnegie et al. (2013) suggests that *B. roughleyi* is not a *Bonamia* sp. at all, and finally *Bonamia perspora*, which parasitizes *Ostreola equestris* in North Carolina (Carnegie et al. 2006). Several unidentified forms of *Bonamia* have been found in other oysters species over the last decade, including, *Crassostrea ariakensis* in North Carolina (Burreson et al. 2004), Suminoe oyster *Crassostrea rivularis* reared in France (Cochennec et al. 1998),

Tiostrea chilensis in Chile (Campalans et al. 2000) and in *Ostrea puelchana* in Argentina (Kroeck & Montes 2005).

B. ostreae parasitizes the haemocytes that circulate to all tissues of *O. edulis* (Culloty & Mulcahy 2007). The parasite then multiplies by binary fission within the cells (Poder et al. 1983). Several different forms of *B. ostreae* have been observed by light and electron microscopy. Uninucleate, highly basophilic, dense cells are seen most frequent. They are spheroid in shape, measure 2-3 µm in diameter, and contain a nucleus surrounded by a pale halo. Uninucleate slightly basophilic, light cells are seen less frequently. They are somewhat elongated and measure 3-5 µm. Binucleated cells and multinucleate plasmodial forms are also observed. The plasmodial stage can be as large as 6 µm in diameter and contain from 3 to 5 nuclei. This stage develops sporadically (Grizel et al. 1988, Culloty & Mulcahy 2007, Lallias et al. 2008).

B. exitiosa parasitizes host haemocytes and also displays haplosporosome-like structures and multinucleate plasmodial cell forms (Hine et al. 2001). *B. exitiosa* was considered a southern hemisphere *Bonamia* species until its discovery in *O. edulis* samples taken from the Galician Coast, Spain, in 2007 (Abollo et al. 2008), in the Adriatic sea, Italy (Narcisi et al. 2010), in the Mediterranean sea off Spain (Carrasco et al. 2012) and most recently in south western England (Longshaw et al. 2013). These findings confirm that *B. exitiosa* is no longer a southern hemisphere species and furthermore confirms a new host for the parasite. The possible occurrence of *B. exitiosa* infection may not have as significant an impact on native *O. edulis* populations as was previously thought. Low levels of mortality occurred on the English population upon infection, which Longshaw et al. (2013) speculated was due

to an intolerance of the parasite to lower temperatures. Ireland has a similar climate to England thus temperature limitations would also apply here.

B. roughleyi, which parasitizes *S. glomerata* in Southeast Australia, is, again, very similar to *B. ostreae* in behaviour and appearance (Cochennec et al. 2003). *B. perspora*, unlike other *Bonamia* species, possesses far more diverse cell forms. Besides producing the uninucleate, binucleate, and plasmodial stages retained by other *Bonamia* spp., *B. perspora* also produces sporonts, sporocysts, and spores thus becoming the first known member of the *Bonamia* genera to display a sporulated life stage (Carnegie et al. 2006).

A new microcell parasite was uncovered in Bogue Sound, North Carolina after severe mortalities occurred in triploid *C. ariakensis* stocks produced in a Virginia hatchery (Burreson et al. 2004). A small study of the subunit ribosomal gene confirmed it to be a new *Bonamia* species, one especially pathogenic to triploid *C. ariakensis* under 50 mm in length (Burreson et al. 2004). The parasite has since been observed in more *C. ariakensis* stocks distributed in several other sites in North Carolina (Carnegie et al. 2008). Unlike other *Bonamia* species, the *Bonamia sp*. in *C. ariakensis* appears to have an inclination towards euhaline water only (Bishop et al. 2006) and displays a very strong seasonality, peaking in late spring, summer and becoming virtually non-existent in late autumn and winter (Carnegie et al. 2008).

Extensive mortalities of cultured *O. puelchana* stocks were reported in San Antonio Bay, Argentina during 1996. A *Bonamia* sp. was identified as the source. Its correct taxonomy is still to be determined, but gross signs and histopathology in *O. puelchana,* as well as cytology and developmental stages of the *Bonamia* sp. resemble those reported for *B. exitiosa*. Until further molecular analysis is carried out, its classification remains unknown (Kroeck & Montes 2005).

Phylogeny of *Bonamia ostreae*

Bonamia ostreae's inclusion into the phylum Haplosporidia has been confirmed molecularly by two studies, Carnegie et al. (2000) and Cochennec et al. (2000). Initially, *B. ostreae* was considered to be of uncertain taxonomic position though Balouet et al. (1983) believed that this parasite was the early stage of a haplosporidian. It is assigned to the taxonomic group, Haplosporidia, by the presence of haplosporosomes and on the basis of molecular analysis of the SSU rRNA gene, though a spore stage has never been witnessed in any *B. ostreae* infections (Carnegie et al. 2000, Cochennec et al. 2000). Bonami et al. (1985) suggested that its developmental cycle might not include a spore stage in this species. Moreover a spore stage may be finally observed when the parasite is found in its natural host (Carnegie et al. 2006). *Bonamia* have been included in a phylum of spore-forming protists with presumably complex life cycles as *Bonamia* is now recognized as a spore-forming genus that includes several species that may have abandoned production of spores (Carnegie et al. 2006).

Throughout the years there have been several taxonomic arrangements proposed for the placement of the group within the protists and for appropriate taxa within the Haplosporidia. Caullery & Mesnil (1899) originally established Haplosporidium for two parasites of annelids, placing the genus in the new order Haplosporidia in the class Sporozoa of the phylum Protozoa. Caullery (1953) recognized 6 genera in the order, while Kudo (1971) recognized 7 genera. In his 1979 paper, Sprague proposed the separation of the Haplosporidia and the Paramyxea from other members of the class Sporozoa by establishing a new phylum named Ascetospora with two classes; Stellatosporea, for the families Marteiliidae and Haplosporidiidae, and Paramyxea,

for the family Paramyxidae. The family Haplosporidiidae contained only 3 genera, i.e., *Haplosporidium*, *Minchinia*, and *Urosporidium*.

Desportes & Nashed's (1983) paper suggested two classes in the phylum Ascetospora - Haplosporea and Paramyxea. At the recommendation of Cavalier-Smith (1993) the phylum Ascetospora has now been abandoned, and Haplosporidia and Paramyxea have each been elevated to phylum rank. Analysis by Berthe et al. (2000) provided molecular phylogenetic support for the notion of separate phylum rank for Haplosporidia and Paramyxea. The most recent phylogenetic analysis involving the Haplosporidia (Cavalier-Smith & Chao 2003) hypothesizes that the group includes the Paramyxea and falls within the Cercozoa.

Sequencing of the SSU rRNA gene of *B. ostreae* in the late 1990s made genetic analyses possible (Carnegie & Cochennec-Laureau 2004). More recently isolation of the gene coding for the small subunit of the ribosomal RNA in *B. ostreae* (18S or SSU rDNA) continued to clarify the phylogenetic position of *Bonamia* species especially *B. ostreae* (Carnegie et al. 2000). SSU rDNA sequence of *B. ostreae* by Cochennec et al. (2000) revealed a strong similarity with *Haplosporidium nelsoni, Haplosporidium costale,* and *Minchinia teredinis,* suggesting that *B. ostreae* shares homologies with the Haplosporidia. Moreover parsimony analysis on SSU rRNA genes placed *B. ostreae*, with strong (100% bootstrap) support, in the Haplosporidia, with *H. nelsoni*, *H. costale*, and *M. teredinis* as its closest relatives (Carnegie et al. 2000). In López-Flores et al. (2007) the genes for coding actin, a cytoskeletal protein involved in cellular functions, such as maintaining cell morphology, cell motility and division, and intracellular transport, were used for phylogenetic analysis of *B. ostreae* at a different evolutionary rate to ribosomal genes. This analysis placed *B. ostreae* in a clade with *Minchinia tapetis, M. teredinis* and *H. costale* as its closest

relatives. Further actin analysis based on amino acid sequences, placed *H. nelsoni* as the closest species to *B. ostreae*, in a sister clade to that formed by *H. costale, M. tapetis* and *M. teredinis* (López-Flores et al. 2007).

In recent years, molecular phylogenetic analyses have also been used to confirm *Bonamia exitiosa* and *Bonamia roughleyi'*s relationship to *B. ostreae*. Analysis of the SSU rDNA of *B. exitiosa* and *B. ostreae* found that 96.6% were similar over 1623 bp (Hine et al. 2001). Then using parsimony analysis on *B. roughleyi,* Cochennec-Laureau et al. (2003b) determined that the SSU rDNA sequence had 95.2% similarity with *B. ostreae* and 98.4 % with *B. exitiosa* placing *B. roughleyi* with *B. ostreae and B. exitiosa* in the Haplosporidia. Carnegie & Cochennec-Laureau's (2004) SSU rDNA sequences of *B. exitiosa* and *B. roughleyi* were 99.5% similar. The similarity in sequences implies that both species may share a common ancestor subsequent to their divergence from *B. ostreae* (Carnegie & Cochennec-Laureau 2004).

Burreson & Reece's (2006) research into the spore formation of *B. perspora* confounds the distinction between *Bonamia* and *Haplosporidium*. The results suggested that the accepted practice of assigning all species with spore wall-derived ornamentation to *Haplosporidium* cannot be supported and that additional genera are needed in which to place some species presently assigned to *Haplosporidium*.

Geographic distribution of the disease

Having first being identified as "microcell" parasites in California in 1969 (Katkansky et al. 1969), *Bonamia ostreae* was identified in Europe, a decade later, in the *Ostrea edulis* beds of southern Brittany, France, which had started to display a high rate of mortality (Comps et al. 1980, Tigé et al. 1980). It is now believed that

the disease spread from the US to France through the commercial import of spat from American hatcheries (Elston et al. 1986, Grizel et al. 1988). Mialhe et al. (1988) found no antigenic differences between the Californian and French samples when they were examined using monoclonal antibodies. The disease subsequently spread throughout Europe and parts of the US during the 1980s.

B. ostreae was found in Asturias, Spain in 1980 (Figueras 1991). Cigarría & Elston (1997) suggested that the infection in Spain may have also originated from California and thus the disease had two sources in Europe. Holland was the next confirmed country of infection, after infected stock from France was imported into Yerseke Bank in 1980 (Balouet & Poder 1983). By 1988 it had spread to Lake Grevelingen (van Banning 1990) even though measures were introduced to control the spread of the disease from Yerseke Bank (van Banning 1991). Several attempts were also made to eradicate the parasite from Yerseke Bank but none were successful (van Banning 1985, 1987). Bonamiosis was discovered in England in the autumn of 1982, having caused large mortalities in the oyster beds of the rivers Fal and Helford in Cornwall (Bannister & Key 1982, Bucke & Feist 1985). As with Holland, the UK had introduced measures to prevent its introduction and spread which failed and soon the parasite had spread all over the south and east coast (Laing et al. 2006).

Again the movement of infected Californian stock was thought to be the cause of the spread of *B. ostreae* throughout the USA (Elston et al. 1986, Friedman & Perkins 1994, Cigarría & Elston 1997). In the mid 1980's Bonamiosis was found in several populations in Washington state on the West coast (Elston et al. 1986). By the early 1990's it was found to have spread to Maine on the eastern coast (Barber & Davis 1994, Friedman & Perkins 1994, Zabaleta & Barber 1996). Several additional

countries have been identified as housing *B. ostreae* infected areas since the turn of the century. *B. ostreae* was detected in British Columbia on the west coast of Canada in 2004 (Marty et al. 2006). It was detected in Morocco in 2005 while in 2006 it was identified in Scotland (Murray et al. 2012) and Wales (http://www.cefas.defra.gov.uk/idaad/abstract.aspx?t=rt&id=1017). The last outbreak of *B. ostreae* was in Norway in 2009 (http://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?page_refer=MapFullEventReport&reportid=8166).

Pathology of bonamiosis

The initial progression of *Bonamia ostreae* infection within *Ostrea edulis* remains unknown, at present, as uninfected oysters exposed to the parasite display no clinical sign of infection within their cells for between 4 weeks and up to several months post infection, the so-called "latent" stage of infection (Tigé & Grizel 1984, Elston et al. 1987, Montes 1991, Culloty & Mulcahy 1996, Culloty & Mulcahy 2001). It is not possible to detect the parasite in the host's tissues with either heart imprints or histology-based techniques during this latent period (Poder et al. 1982).

One mechanism that *B. ostreae* may use to enter a flat oyster is through the gill epithelia (Montes et al. 1994) but access may also occur during feeding by the oyster. Subsequent to the latent period, *B. ostreae* parasitizes the haemocytes (Culloty & Mulcahy 2007). The parasite multiplies by binary fission within the infected haemocytes eventually leading to the rupture of the cell and the further spread of the parasite throughout the tissue (Poder et al. 1983, Culloty & Mulcahy 2007). Eventually the parasite can be observed extracellularly between epithelial or interstitial cells in gills and stomach or in necrotic connective tissue areas (Arzul et

al. 2009). Infection with *B. ostreae* often results in massive inflammation and the death of its host (Bucke & Feist 1985). Infected animals may appear normal, though yellow discolouration and/or extensive perforated ulcers have been observed in the connective tissues of the gills, mantle and digestive gland (Balouet et al. 1983). This appears to have been more prevalent during the early stages of infection in naïve stocks. Heavily infected oysters also tend to be in poorer condition than uninfected oysters (Bower 2007). The disease can be devastating to oyster stocks, particularly when the parasite initially enters a stock, with infections being associated with 80% + mortalities within 6 months of exposure to a naïve oyster population (Poder et al. 1982, Balouet et al. 1983, Friedman & Perkins 1994, Culloty & Mulcahy 2007).

Immune response of *Ostrea edulis* to infection

Haemocytes play a pivotal role in molluscan defence against pathogens (Morga et al. 2009). For *Ostrea edulis,* haemocytes have been classified into three groups based on morphology; granulocytes, large hyalinocytes and small hyalinocytes (Auffret 1989, Chagot 1992, Mourton 1992, Xue & Renault 2001, Morga et al. 2009). *Bonamia ostreae* and some other haplosporidians are more frequently phagocytosed by granulocytes (Balouet & Poder 1985, Mourton et al. 1992) though both granular (granulocytes) and agranular (hyalinocytes) blood cells types can be infected by the parasite (Culloty & Mulcahy 2007). Ordinarily phagocytosis of pathogens by haemocytes is the main cellular immune response of bivalve molluscs to a disease (Cheng 1981, Carballal et al. 1997, López et al. 1997). Once the pathogen have been internalised in the haemocyte, the blood cell will use various mechanisms to kill the pathogen (Culloty & Mulcahy 2007).

In the case of bonamiosis, the parasite is internalised by the haemocytes yet the haemocytes fails to kill the parasite, allowing for multiplication of the parasite within the cell, leading to haemocyte rupture and infiltration of the parasite throughout the oyster tissues (Chagot et al. 1992, Mourton et al. 1992, Montes et al. 1994). Hervio et al. (1991) reported that the parasite possesses catalytic enzymes and acid phosphatase, which could inhibit parasite degradation within the haemocyte. Morga et al. (2009) and (2011) also discovered that *B. ostreae* infection within a haemocyte decreased non-specific esterase activities and reactive oxygen species production by the haemocyte and genetically expressed sequence tags induced an increased expression of omega glutathione S-transferase, superoxide dismutase, tissue inhibitor of metalloproteinase, galectin, interferon regulatory factor and filamin genes. Prado-Alvarez et al. (2013) hypothesize that the *B. ostreae* heat shock protein 90 could be involved in the infection process of *O. edulis* haemocytes.

B. ostreae can influence the distribution of haemocyte types, reducing the number of granulocytes through destruction or degranulation, as they fight infection (Cochennec-Laureau et al. 2003a). A study comparing the reaction of *O. edulis* and *Crassostrea gigas* to *B. ostreae* exposure showed significant differences in total haemocyte count, differential haemocyte count and respiratory burst between the two species, which could be linked to differences in susceptibility to bonamiosis (Comesana et al. 2012).

Epidemiology of *Bonamia ostreae*

Bonamia ostreae occurs throughout the year, yet many studies have observed a seasonal variation in infection, therefore temperature may be one factor affecting the spread of the disease (Grizel & Tigé 1982, Cáceres-Martínez et al. 1995, Culloty &

Mulcahy 1996, Engelsma et al. 2010). The majority of studies found prevalence of infection peaked in late winter and in autumn, often associated with post spawning stress (Grizel 1985, Montes 1990, van Banning 1991, Culloty & Mulcahy 1996, Arzul et al. 2006). In Holland, the highest prevalence was recorded in spring, but parasite prevalence appeared to be higher after a warm autumn (Engelsma et al. 2010). Arzul et al. (2009) noted that water characteristics, such as temperature and salinity, could affect the infective capacity and spread of *B. ostreae.* Cochennec & Auffret's (2002) study of *B. ostreae* prevalence showed that prevalence was higher at the lower temperature of 10°C compared to 20°C. A more recent study showed that there was a significantly lower survival at 25°C compared to 15°C, with good survival to 4°C (Arzul et al. 2009). In addition, the disease was confirmed for the first time in a population in Norway in June 2009 (http://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?page_refer=MapFullEventReport&reportid=8166), indicating that low temperatures may not have the same limiting effect on the disease as high temperatures.

The presence of *B. ostreae* infection in *Ostrea edulis* has also been associated with age (Grizel 1985). Initial studies suggested that older (2 years +) flat oysters were more susceptible than younger oysters (Grizel & Tigé 1982, van Banning 1990, McArdle et al. 1991, Robert et al. 1991, Culloty & Mulcahy 1996, Engelsma et al. 2010) yet other studies have shown pre-spawning oysters (Lynch et al. 2005) and larvae (Arzul et al. 2011) can also be infected by the disease, indicating that all ages are susceptible. da Silva et al. (2009) proposed that older and larger oyster had a higher probability of capturing infective particles through longer exposure. In addition, some physiological changes, such as gonadal condition, dependent on age, could favour the progression of the infection.

In terms of the link between sex of the oyster and *B. ostreae* infection, earlier studies, such as van Banning (1990), suggest that the oyster gonad cycle influences the infection and that *B. ostreae* has an incubation period in the oyster ovary before the next stage develops in the haemocytic phase, yet in subsequent studies the disease has been discovered in males and no correlation has been found between *B. ostreae* prevalence and gonadal development (Cáceres-Martínez et al. 1995, Culloty & Mulcahy 1996). da Silva et al. (2009) suggested that there is a link between intensity of infection and sex, in that intense infections have a greater probability of occurring in females and even more so in ripe and recently spawned females, due to the weakening of the defence system during gametogenesis. However, overall there has been no consistency on this point between different studies.

Bonamia ostreae in Ireland

Bonamia ostreae was diagnosed in oysters in Ireland in 1987, after large mortalities were observed in the Rossmore beds, in the North channel of Cork harbour, which is situated on the southern coast (McArdle et al. 1991). By the time the disease was diagnosed, mortalities of over 90% were being observed in oysters aged 4+ (McArdle et al. 1991). It is believed that the original infection occurred in 1984, when unusual levels of mortalities began occurring but was not successfully diagnosed till 1987. Frozen samples from 1986 were screened and found positive for the presence of *B. ostreae* lending some proof to this theory (Rogan et al. 1991). The means by which the parasite was introduced into Ireland remains unknown, but unlicensed movement of oysters is suspected (Minchin et al. 1993, Culloty & Mulcahy 2001, Culloty & Mulcahy 2007).

Within a year of its detection in Cork harbour, the disease had spread to the west coast, confirmed in a consignment of oysters from the wild beds of Clew Bay, Co Mayo (McArdle et al. 1991). *B. ostreae* was diagnosed in one bed in Galway Bay in 1989. By 1991 it had spread to five new sites within the bay (McArdle et al. 1991). By 1994 the infection had spread to Achill Sound, Co Mayo. Spread of the disease appeared to slow until 2003 when the neighbouring Blacksod Bay was confirmed to be infected. In 2005, Lough Foyle, on the north coast of the country, was found to be infected, quickly followed in 2006 by the confirmation of the disease in nearby Lough Swilly (Culloty & Mulcahy 2007). The national screening agency, the Marine Institute, concluded that the disease may have been spread to the north coast through the unauthorised movement of mussels, containing oyster spat, from a non-approved zone in the west of the country (Marine Institute et al. 2006). As of today, only Tralee Bay, Co. Kerry and Kilkieran Bay, Co. Galway are *B. ostreae* free (Culloty & Mulcahy 2007).

Potential hosts of the parasite

The complete life cycle of *Bonamia ostreae* may consist solely of direct transmission from flat oyster to flat oyster (Elston et al. 1986, Hervio et al. 1995, Culloty et al. 1999), which is easily demonstrated in the laboratory and is atypical of a haplosporidian. The ability of *B. ostreae* to survive in areas with low to very few oysters being present would indicate that other carriers or life stages of the parasite may exist. This was supported by van Banning's (1988) research, where oyster beds were dredged and left fallow for a number of years followed by the reintroduction of naïve, uninfected oysters, which rapidly became infected.

It is possible that a macroinvertebrate species may act as either an intermediate host or reservoir in the life cycle of *B. ostreae*. Lynch et al. (2007), using a molecular based approach, found eight benthic macroinvertebrates and nineteen grouped zooplankton samples gave positive results for the presence of *B. ostreae* when screening took place in Cork harbour over a period of 6 months. In subsequent cohabitation trials, transmission of *B. ostreae* occurred from the Brittlestar *Ophiothrix fragilis* to cohabiting naïve (i.e. previously uninfected) flat oysters. Several previous studies had also looked at the susceptibility of other bivalve species and their ability to act as carriers, but had failed to detect the presence of *B. ostreae*, possibly as all screening was based on histological and cytological techniques (van Banning 1987, Figueras & Robledo 1994, Culloty et al. 1999).

A controversial species, in terms of acting as a reservoir or carrier of *B. ostreae,* is the Pacific oyster, *Crassostrea gigas,* as the species is frequently farmed alongside *Ostrea edulis*. At present, *C. gigas* is classed as a non-carrier species by the European Commission, thus it can be exported to disease free zones. Renault et al. (1995) and Culloty et al. (1999) attempted and failed to induce an infection in *C. gigas,* either by inoculation or by cohabitation with infected *O. edulis*. Lynch et al. (2010) screened *C. gigas* using molecular and histological techniques and detected *B. ostreae* DNA in the tissues and shell cavity fluid of a small number of oysters in the field and in the laboratory, while *B. ostreae*-like cells were observed in the cells of two oysters. The Spanish co-authors on the study also detected *Bonamia exitiosa* DNA within *C. gigas* tissue, indicating that in this species, a small number of individuals could act as a carrier of both *B. ostreae* and *B. exitiosa.*

Diagnosis of *Bonamia ostreae*

The diagnostic techniques recommended by the OIE are the histological examination of tissue sections, the screening of heart/gill imprints and genetic based techniques such as Polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and *in-situ* hybridisation (ISH).

The production of a ventricular heart imprint allows the intensity of infection in the blood cells to be determined (Bachere et al. 1982, Culloty et al. 2004). Histology allows the spread of the parasite throughout the tissue to be observed (Carnegie et al. 2000, Culloty et al. 2003, Diggles et al. 2003, Marty et al. 2006) and the overall health status of the oyster to be assessed (Diggles et al. 2003, Balseiro et al. 2006, Culloty & Mulcahy 2007, Lynch et al. 2008). Genetic analysis allows small quantities of parasite DNA to be detected (Carnegie et al. (2000) while *in-situ* hybridisation, combining both histological and genetic methods, allows the visualisation of very low numbers of parasites within the oyster and allows the study of cryptic or early stages of the parasite (Culloty & Mulcahy 2007).

Fluorescent *in-situ* hybridisation (FISH) is a similar technique to *in-situ* hybridisation, whereby infected cells and pathogens are identified by fluorescence in preference to the colour change of ISH. Carnegie et al. (2003) found it to be more specific than the ISH described by Cochennec et al. (2000), which also detected *Bonamia exitiosa* and *Haplosporidium nelsoni*. Its primary weakness is its cost (Carnegie et al. 2003).

Due to the margin of error possible with all of the procedures, many studies recommend the combination of a microscopy based method with molecular techniques (Walker & Subasinghe 2000, Culloty & Mulcahy 2007, Lynch et al. 2008). Presently the OIE (2010) recommends the use of either histology or heart

imprints for diagnosis of the disease, while PCR is advised for confirmatory identification. Recently new species-specific conventional PCR and real-time PCR techniques were developed to diagnose *B. ostreae* and *Bonamia exitiosa* (Ramilo et al. 2013).

Methods of control and management of bonamiosis

At present no method exists to eradicate the disease from an area once infection has occurred, van Banning (1987) found that even after the removal of the majority of flat oysters from the area and a fallowing period of several years, oysters reintroduced into Lake Grevelingen, the Netherlands, developed bonamiosis. Culture of flat oysters in Europe and the US is now governed by measures to control the threat of *Bonamia ostreae* (Kennedy & Roberts 1999). In Ireland, all of the areas where *B. ostreae* was detected continue to be infected by the disease. Only two areas in Europe are considered to have possibly eradicated the disease. There were reports of *B. ostreae* in the Limfjord region of Denmark but these infections were in foreign oysters being held temporarily in the area (Peterson *pers comm*.). In Scotland, efforts have been made to rid Loch Sunart of *B. ostreae* by removing all oysters (Bland & Fraser 2012).

A selection of husbandry techniques may minimise mortalities from the disease (Culloty et al. 2004) including reducing stocking densities, fallowing beds, on-growing in deeper waters, using suspension culture, cleansing sites and fishing gear, culling lays in which infestation levels exceed 10% and marketing oysters after 15-18 months (Grizel et al. 1988, Lama & Montes 1993, Hugh-Jones 1994, Montes et al. 2003).

OIE and EU legislation on *Bonamia ostreae*

With regards to control of the disease, the OIE International Aquatic Animal Health Code have put in place clear measures to prevent any further spread of the disease (OIE 2010). *Bonamia ostreae* is classified as a notifiable disease by the OIE and in turn the European Union (EU), under the Council Directive 2006/88/EC, list II of Annex A (www.eur-lex.europa.eu), indicates that declaration of an occurrence of the disease is compulsory. Also as a notifiable disease, movement of *B. ostreae* infected stock is not permitted. As a result of this, all countries within the EU are required to undertake national screening programs to determine and monitor the disease status of their *Ostrea edulis* populations. If an area is considered infected with bonamiosis, exportation of flat oysters to areas, which have an equal zoosanitary status, may continue, but exportation to disease-free areas is heavily limited, with any exported stock to remain in complete isolation from the native disease-free populations. Upon certification of their status, disease-free stocks can be exported without any such limitations.

To prevent the further spread of the disease within a country once an infection has occurred, the EU established a zoning system with increased movement controls and an intensified degree of surveillance. Zones can subsequently be declared *B. ostreae* free once two years has passed without any further detection of *B. ostreae.*

With the discovery that oyster species other than *O. edulis* may act as hosts for *B. ostreae* (Le Borgne & Le Pennec 1983, Bougrier et al. 1986, Bucke & Hepper 1987, Pascual et al. 1991, Cochennec et al. 1998) these regulations now also apply to Australian mud oysters (*Ostrea angasi*), Argentinean flat oysters (*Ostrea puelchana*), Chilean flat oysters (*Ostrea chilensis*), Asiatic oysters (*Ostrea denselammellosa*) and Suminoe oysters (*Crassostrea ariakensis*).

Selective breeding for resistance to *Bonamia ostreae*

As attempts to control the disease have been only marginally successful, the OIE suggests the breeding of a "resistant" strain of *Ostrea edulis,* which may act as an alternative option in already infected areas. Ford (1986) describes resistance as greater survival, implying a reduced susceptibility to the presence of the parasite, which allows the oysters to be grown to market size before disease-induced mortalities occur. Genetically distinct populations of the flat oyster still exist along the European coastline, in spite of numerous oyster transfers carried out over the last century (www.fao.org/fishery/culturedspecies/Ostrea_edulis). Programmes to produce resistant strains started with IFREMER in France being the first in 1985 (Naciri-Graven et al. 1998) with the survivors of infection (Mialhe et al. 1988) being used to produce the next generation (Launey et al. 2001). However, inbreeding occurred within the strains (Naciri-Graven et al. 1998, Launey et al. 2001) but reports indicated a survival rate of 59% in the third generation compared to 13% for control wild-type oysters in *B. ostreae*-contaminated areas (Baud et al. 1997). However, more recently there have not been any further updates on these strains or their performances. However it would appear that in wild populations, particularly in Brittany, prevalence and intensity of infection have decreased significantly in oyster stocks now held at much lower densities (Arzul at al. 2006).

In Ireland, after the discovery of *B. ostreae* in their flat oyster stocks in Rossmore, Co. Cork, "Atlantic Shellfish Ltd." established a breeding program, in 1988, to produce a disease resistant stock. Oysters, 4+ years, which had survived the initial infection were used as broodstock. Land-based spatting ponds were used for controlled spawning and oysters were then on-grown on specific beds (Culloty & Mulcahy 2001). Subsequent generations and their performance and resistance to *B.*

ostreae were assessed within Ireland (Culloty & Mulcahy 2001, Culloty et al. 2004). The "resistant" stock showed lower prevalences of *B. ostreae* infection and lower intensity relative to the naïve oysters while also exhibiting fewer mortalities. The studies did indicate that populations of oysters may develop local adaptions to factors, such as environmental conditions, making them unsuitable for transfer to sites of different conditions.

The advances in genetics that have occurred in recent years, will allow for improved breeding of *B. ostreae* resistant oyster stocks, as microsatellites and then quantitative trait locus' may be mapped onto *O. edulis* chromosomes, and genes subsequently identified that underlie the tolerance or susceptibility to bonamiosis (Carnegie & Cochennec-Laureau 2004). Recently, a group of expressed sequence tags that play a role in the immune response, such as cytokines, stress proteins, eicosanoids and proteins implicated in phagocytosis were identified, and their expression was analysed at different infection levels by Q-PCR to aid in identifying *B. ostreae* resistant oysters (Martín-Gómez et al. 2011). A new study suggests that apoptosis plays a key role in the resistance of flat oysters to bonamiosis. *B. ostreae* can induce the expression of the anti-apoptotic gene OeIAP, inhibiting apoptosis, to allow for survival inside the haemocytes, while resistant oysters can eliminate or limit the development of the parasite by inducing apoptosis through the decrease of cyclophilin (Morga et al. 2012).

Oysterecover

Due to the disease caused by *Bonamia ostreae*, *Ostrea edulis* is now listed by OSPAR as a threatened or declining species (Haelters & Kerckhof 2009), thus conservation of this oyster species is now required. Some measures and plans are

being undertaken such as the National parks and wildlife services conservation objectives for special areas of conservation (www.npws.ie/protectedsites/conservationmanagementplanning) and the European Union funded Oysterecover project.

The research conducted in this thesis was carried out as part of the Oysterecover project, which united research centres and universities with small and medium sized shellfish producers. The title of the project was "Establishing the scientific bases and technical procedures and standards to recover the European flat oyster production through strategies to tackle the main constraint, bonamiosis". The main objective of the project was to look at aspects of the disease bonamiosis, caused by the parasite *B. ostreae,* with the ultimate objective of minimising the impact of the disease and helping to conserve *O. edulis*. In addition to the research presented here, studies were also conducted on the interactions between the parasite and the host haemocyte and the genetic basis for flat oyster tolerance to bonamiosis. The eight research institutes and universities involved included Cefas, UK, IMARES, the Netherlands and the Universidade de Santiago de Compostela, Spain. The seven shellfish producers involved included Clew Bay Oyster Co-operative Society Ltd., Nederlandse Oestervereniging and Atlantic Shellfish Ltd.

The parts of the project to be concentrated on, in this study, were validation of diagnostics, studies on aspects of the life cycle of *B. ostreae* and how it maintains itself within the environment and assessing oyster stocks with varying histories of exposure to the disease in the field to assess current health status and in the laboratory to determine how the varying histories of exposure may impact on the progression of infection in the weeks following exposure.

Objectives

The main objectives of this study were:

1. To compare the sensitivity of the most commonly used screening tools for the diagnosis of *Bonamia ostreae,* in order to establish how effective these tests are in detecting the disease. In Chapter 2, all four methods of *B. ostreae* diagnosis recommended by the OIE were compared within and between three laboratories, to assess reproducibility of results amongst laboratories.

2. In Chapter 3 – Part A, natural oyster stocks from Clew Bay and Lough Foyle, Ireland, affected by *B. ostreae* for varying periods, were monitored for a period of 13 months to determine whether exposure to *B. ostreae,* over different periods of time, in wild, unmanaged flat oyster stocks would result in changes in the susceptibility or resistance in these stocks. In addition the role of environmental and stocking factors on disease levels were assessed.

3. To determine whether different stocks of *O. edulis* with differing histories of exposure to *B. ostreae* can be used to restock areas affected by *B. ostreae* infection. In Chapter 3 – Part B, spat from three oyster stocks from the North Sea region with different histories of exposure to *B. ostreae* – Lake Grevelingen and the Oosterschelde, both in Holland, and Limfjord, Denmark, were placed in the *B. ostreae* infected Lake Grevelingen and monitored, in cages, for a period of 22 months, for prevalence of infection, growth and mortality and to assess overall prevalence within the region.

4. To further expand previous work on how *B. ostreae* might maintain itself on an oyster bed, over an extended period of time, even at low oyster densities. In Chapter 4 – Part A, sediment and macroinvertebrates collected from areas where *B. ostreae* is present were screened for infection to determine if they could act as

reservoirs or incidental carriers of *B. ostreae*. A laboratory based transmission trial was also carried out; to establish if sediment from an infected area might carry the parasite life stages and so could transmit the disease to naïve oysters.

5. In Chapter 4 - Part B, PCR analysis was used to determine how significant the issue of infection in larvae is and the see if brooding larvae infected with *B. ostreae* acquired the infection vertically from the parent or if the source of infection might be from environmental sources. The work was carried out by screening parents and larvae from infected stocks.

6. To gain an understanding of the "latent" stage of infection by determining the progression of *B. ostreae* cells within *Ostrea edulis,* after initial infection, from three stocks with different exposures to the disease. This progression was investigated in Chapter 5, using previously exposed stocks from Clew Bay and Lough Foyle, Ireland and a naïve stock from Loch Ryan, Scotland. All three stocks were exposed to *B. ostreae* cells in the laboratory to assess how differing histories of exposure might impact on the development of infection and progression of the parasite through the oyster tissue.

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Chapter 2

Interlaboratory variability in screening for *Bonamia ostreae*, protistan parasite of the European flat oyster *Ostrea edulis*

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Interlaboratory variability in screening for *Bonamia ostreae*, protistan parasite of the European flat oyster *Ostrea edulis*

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Abstract

The spread of the protozoan parasite *Bonamia ostreae* is of major concern to the European flat oyster industry. Many studies have looked at the sensitivity of individual methods available to screen for *B. ostreae,* but in this study, three separate laboratories examined four methods of diagnosis currently used routinely in laboratories - heart imprints, histology, polymerase chain reaction (PCR) and *in-situ* hybridisation (ISH). The results were compared to estimate interlaboratory variability. Heart imprints and histology had the highest reproducibility amongst the three laboratories with greatest agreement between detection of infected and uninfected individuals. PCR had the highest detection level in every laboratory. These positives were related to the presence of confirmed infections but also, in unconfirmed infections, possibly due to the presence of traces of *B. ostreae* DNA in oysters where clinical infections were not observed. PCR, in combination with histology or ISH, provided the highest detection levels in every laboratory. Variation in results for PCR and ISH observed between the laboratories may be due to the different protocols used by each laboratory for both methods. Overall, the findings from the three laboratories indicated that at least two methods, with fixed protocols, should be used for the accurate detection and determination of prevalence of infection within a sample. This combination of methods would allow for a clearer and more precise diagnosis of *B. ostreae*, preventing further spread of the disease and providing more accurate detection levels and epidemiological information.

Introduction

The sustainability of the European flat oyster industry depends on the development of rapid and accurate diagnostic methods for the monitoring of *Bonamia ostreae*, and the more recently discovered *Bonamia exitiosa*, to prevent the transfer of infected stock. It is essential to limit the transfer of this pathogen as once it is introduced into a population eradication is impossible (van Banning 1987). At present the main foundation of disease control by the Office International des Epizooties (OIE) is certification of stock as *B. ostreae* free prior to movement. The diagnostic techniques recommended by the OIE are the screening of tissue imprints, the histological examination of tissue sections and genetic based techniques such as polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and *in-situ* hybridisation (ISH) (OIE 2012). Screening techniques should be selected primarily based on reliability and sensitivity, however, ease of use, safety and cost should also be considered (Culloty & Mulcahy 2007).

The production of a heart imprint allows screening of the parasite in blood cells and determination of the intensity of infection. Heart imprints are economical and rapid to make in comparison to the other techniques commonly used (Zabaleta & Barber 1996, O'Neill et al. 1998, Carnegie et al. 2000, Culloty et al. 2003, Culloty & Mulcahy 2007, Lallias & Arzul 2008). The major disadvantages to the use of heart imprints is that *B. ostreae*, due to its small size (2–5 μm), may go undetected when the infection is light or in its latent period (Carnegie et al. 2000, Cochennec-Laureau et al. 2003a, Culloty et al. 2003, da Silva & Villalba 2004, Lynch et al. 2008, Bower 2011) and that *B. ostreae* may be confused with other morphologically similar parasites, such as *Mikrocytos mackini* and *B. exitiosa* (Marty et al. 2006, Narcisi et al. 2010).

The main advantage of histology over heart imprints is the ability to make observations on the overall health and physiological status of the oyster (Diggles et al. 2003, Balseiro et al. 2006, Culloty & Mulcahy 2007, Lynch et al. 2008). The primary disadvantages of histology are low sensitivity, misidentification of certain pathogens, difficulty in screening larvae and spat and the length of preparation time (Cochennec et al. 1992, Carnegie et al. 2000, Carnegie et al. 2003, Diggles et al. 2003, Balseiro et al. 2006, Marty et al. 2006).

Standard PCR assays appear to be a more sensitive method of detection than the more traditional, cytological based methods, detecting all classes of infection (Carnegie et al. 2000, Carnegie & Cochennec-Laureau 2004, Balseiro et al. 2006). It can also be used to detect the presence of *B. ostreae* DNA prior to clinical manifestations of the disease and for screening of spat and juvenile oysters (Lynch et al. 2005, Marty et al. 2006). DNA extraction and PCR is fairly rapid, taking 1 – 2 days to complete. Restriction fragment length polymorphism (RFLP) analysis can be used to identify morphologically similar haplosporidians (Hine et al. 2001, Marty et al. 2006). RFLP is at present considered one of the most cost effective molecular markers available (Schlötterer 2004). However, the occurrence of false positives and false negatives is a major issue with PCR (Burreson 2000, Walker & Subasinghe 2000). Unlike histology and cytology, which possess semi-quantitative scales, standard PCR has no form of quantification (Diggles et al. 2003, Lallias & Arzul 2008) thus requiring the use of real-time PCR, a more expensive and complex method.

For the process of *in-situ* hybridisation (ISH), a digoxigenin labelled probe is used to localise a *B. ostreae* specific DNA sequence in a section of oyster tissue. This allows an observer to visualise the parasite and to determine its exact location within the

oyster (Culloty & Mulcahy, 2007). In theory, ISH can be used in the identification of more cryptic stages of *B. ostreae* and other potential carriers of the parasite (Carnegie et al. 2003, Culloty & Mulcahy 2007, Lynch et al. 2008, Lynch et al. 2010). The crucial drawback to the use of ISH is the lack of specificity relating to the OIE mandated probe used in the screening of *B. ostreae*. The probe, as with PCR, currently uses Bo Boas primers. In testing of this method in regions with multiple parasitic infections, the probe attached to *Haplosporidium nelsoni* and *B. exitiosa* (Carnegie & Cochennec-Laureau 2004, Bower 2011). However, in Ireland, where sampling for this study took place, *B. exitiosa* has not been detected to date. *H. nelsoni* has been detected in C*rassostrea gigas* and a single isolated case of *Ostrea edulis* but not in the regions sampled for this study (Lynch el al. 2013).

Though many individual studies have been carried out to assess the sensitivity of each method, few studies have carried out interlaboratory comparisons between methods and samples. In this study, three laboratories with long-term experience of screening for *B. ostreae* carried out an interlaboratory calibration and assessment to look at the reproducibility of four methods for detection of *B. ostreae* and to compare the results when using the same samples of oysters.

Materials And Methods

A sample of *Ostrea edulis,* 60 animals in total, was collected from two *Bonamia ostreae*-endemic locations in Ireland, Clew bay and Lough Foyle, in October 2010 and January 2011, respectively. Final analysis amongst all laboratories resulted in 30 of the 60 animals being screened by all four methods, 17 samples from Clew Bay and 13 samples from Lough Foyle. Three replicates of heart imprints and adjacent tissue sections for histology and ISH were prepared in UCC (Cork, Ireland) for each individual oyster to be subsequently screened by the UCC, CIMA (Vilanova de Arousa, Spain) and Cefas (Weymouth, UK) laboratories. Three small pieces of gill tissue (approximately 5 mm) were also removed from each oyster in UCC to be screened using PCR by each laboratory. All samples were coded to allow for blind testing.

Prevalence and intensity of infection using ventricular heart imprints

Each oyster was opened, the complete heart removed, placed onto tissue paper and the ventricular heart imprinted onto three slides (one slide for each laboratory). The imprints were air dried before being fixed in methanol for two minutes and stained with Hemacolour 2 and 3 (Merck) (Culloty et al. 1999). The imprints were examined in all laboratories under light microscopy at 400x and 1000x magnifications.

Processing and examination of histological sections

For histopathology, an oblique transverse section, consisting of mantle, gills, gonad and digestive gland, was taken from each animal and fixed in Davidson's fixative for 48 hours (Shaw & Battle 1957, Howard et al. 2004). These tissue samples were then embedded in paraffin and cut into 5 μm sections. Finally, sections were placed on

slides (three slides, one for each laboratory) and stained with haematoxylin and eosin. The sections were examined under light microscopy at 400x and 1000x magnifications. Results were categorised as either positive or negative for the presence of *Bonamia ostreae*.

DNA isolation and PCR analysis

Three 5 mm pieces (one piece for each laboratory) of gill tissue were collected from each animal and fixed in 95% ethanol in coded vials. They were then distributed to each laboratory. Different extraction protocols were used in each laboratory, as routinely occurs. In UCC, DNA was extracted from the tissue using the Chelex-100 method (Walsh et al. 1991, Lynch et al. 2010). In CIMA, the commercial kit Wizard Genomic DNA Purification Kit (Promega) was used, according to the manufacturer's protocol. In the case of Cefas, the DNA was extracted from the tissue using the EZ-1 DNA tissue mini kit and an EZ-1 BioRobot (Qiagen), according to the manufacturer's protocol. The extractions were stored at –20°C. In all three laboratories PCR analyses were performed with primers Bo Boas, which amplified 300 bp from the SSU rRNA gene, using the method of Cochennec et al. (2000). All PCR assays included positive controls (*Bonamia ostreae* DNA) and a negative control (double distilled water). The PCR products were run on agarose gel in buffer stained with ethidium bromide. All results were categorised as either positive or negative.

***In-situ* hybridisation**

Tissue sections were placed on silane-prep slides (Sigma Aldrich™), coded and distributed to each laboratory. In each laboratory a probe was generated by PCR

using primers Bo Boas, which target the SSU rDNA gene, and incorporation of digoxigenin during amplification of the 300bp product (Cochennec et al. 2000). In UCC, all solutions and buffers used were made up according to the protocol described by Sambrook and Russell (2001) and Lynch et al. (2008) and cover slipped with DPX mountant (Sigma-Aldrich™). In CIMA, sections were prepared using a similar method to UCC and cover slipped with Histomount (Zymed, Invitrogen).

In Cefas, following preparation of the probe the sections were de-waxed by two immersions in Clearene (Leica Biosystems UK) and then two baths of 99% industrial methylated spirits (IMS). Sections were treated with proteinase K in Tris/NaCl Buffer (TB) and incubated in a moist chamber for 15 minutes before dehydration in 99% IMS. Sections were fitted with Gene Frames (Anachem UK Ltd), overlaid with hybridization buffer containing the digoxigenin-labelled probe and then sealed before being placed in a thermo cycler to denature the probe. Sections were cooled on ice before an overnight incubation at 42 ºC. After Gene frame removal, sections were washed twice in 1 x SSC, 6M Urea, 0.2% BSA in distilled water at 42^0C. Sections were blocked with 6% skimmed milk powder (Fluka, Sigma-Aldrich™) in TB at room temperature and then incubated with anti-Dig conjugated to AP, diluted 1/300 in TB. Sections were placed in five successive washes of 1x TB followed by a brief immersion in 100mM Tris-HCl. NBT/BCIP in alkaline phosphatase buffer was added to the tissue and sections incubated in the dark before immersion in 100mM Tris-HCl. Finally, sections were placed in Nuclear Fast Red solution, rinsed in tap water and dehydrated in 99% IMS before being held in Clearene and cover slipped with Eukitt (Sigma-Aldrich™). All sections were examined under light microscopy at 400x and 1000x magnifications. Results were categorised as either positive or negative.

All four methods of screening were used to determine the percentage of oysters with positive parasite detection within each laboratory. The sets of results from all three laboratories were then compiled and compared.

Results

Percentage of positive samples detected by each method

Heart imprints, histology and ISH were used to determine prevalence of clinical infection following observation of the parasite. Heart imprints had the least variation amongst the laboratories, 33.3 - 36.7% prevalence of infection, while with histology and ISH, prevalences of infection varied from 30-40% and 26.7-36.7%, respectively. PCR, detecting *Bonamia ostreae* DNA, had the highest variation in detection, 46.7-60% (Table 1). Some positive results were obtained with this method in individuals that did not display clinical infection.

Table 1. Percentage of oysters *Ostrea edulis* in which *Bonamia ostreae* was detected using each diagnostic method in each of the three laboratories.

	Heart imprints	Histology	PCR	ISH
UCC	33.3% (10/30)	30% (9/30)	46.7% (14/30)	36.7% (11/30)
CIMA	36.7% (11/30)	40% (12/30)	50% (15/30)	26.7% (8/30)
Cefas	33.3% (10/30)	40% (12/30)	60% (18/30)	33.3% (10/30)

Reproducibility of results for each method

Heart imprint screening had the highest level of reproducibility amongst the three laboratories. Overall, 80% of the results, both positive and negative, from all three laboratories, agreed. Histology followed closely with an agreement of 77%. ISH had an agreement of 63.3%. The least overall agreement was observed using PCR with only 53.3% of the sample results being the same amongst the three laboratories (Table 2 & 3).

Table 2. *Ostrea edulis* in which *Bonamia ostreae* was detected using each diagnostic method in each of the three laboratories with (+) denoting present and (-) denoting not present .

	Heart Imprints			Histology			PCR			ISH		
Oyster no.	UCC	CIMA	Cefas	UCC	CIMA	Cefas	UCC	CIMA	Cefas	UCC	CIMA	Cefas
1	-	-	-	-	-	-	+	+	+	+	-	-
2	+	+	+	+	+	+	-	+	+	+	+	+
3	+	+	+	+	+	+	-	+	+	+	+	+
4	+	+	-	+	+	-	-	+	+	+	-	-
5	+	+	-	+	+	-	-	+	+	+	-	-
6	+	+	+	+	+	+	-	+	+	-	+	+
7	+	+	+	+	+	+	+	+	+	-	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+
10	-	-	-	-	-	-	-	+	-	+	-	-
11	-	-	-	-	-	-	+	-	+	-	-	-
12	-	-	-	-	-	-	-	-	+	-	-	-
13	-	+	-	-	+	+	+	+	+	-	-	+
14	+	-	+	-	+	+	+	+	+	+	+	+
15	-	-	-	-	-	-	+	-	-	-	-	-
16	-	-	-	-	-	+	+	-	-	-	+	-
17	-	-	-	-	-	-	-	-	-	+	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	+	+	-	-	-	-	-	+
20	-	-	-	-	-	-	-	-	+	-	-	-
21	-	-	+	-	-	-	+	-	-	-	-	-
22	-	-	-	-	-	-	-	+	+	-	-	-
23	-	+	-	-	-	+	+	+	+	+	-	+
24	-	-	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	-	-	-	-
27	-	-	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	+	-	+	-	-	-
29	-	-	-	-	-	-	+	-	-	-	-	-
30	+	+	+	+	+	+	+	+	+	+	-	-

Table 3. Agreement of results for all oysters, both positive and negative diagnosis, among the three laboratories.

	Heart imprints	Histology	PCR	ISH
UCC/CIMA	90% (27/30)	90% (27/30)	60% (18/30)	70% (21/30)
UCC/Cefas	90% (27/30)	77% (23/30)	63.3% (19/30)	70% (21/30)
CIMA/Cefas	80% (24/30)	87% (26/30)	83.3% (25/30)	93.3% (28/30)
UCC/CIMA/Cefas	80% (24/30)	77% (23/30)	53.3% (16/30)	63.3% (19/30)

Detection rate with combined diagnostic methods

A combination of methods by pairs increased the percentage of positive cases. In the UCC laboratory, the combination of PCR + ISH had the highest overall detection of *Bonamia ostreae* (66.7%) followed by PCR + histology and heart imprints + PCR (63.3%). The combination of PCR + histology and PCR + ISH had the highest overall detection of *B. ostreae* (53.3%) at CIMA followed by heart imprints + PCR (50%). The combination of PCR + histology had the highest overall detection of *B. ostreae* (66.7%) at Cefas followed by PCR + ISH (63.3%) and heart imprints + PCR (63.3%). Heart imprints + histology had the lowest overall detection of *B. ostreae* in all three laboratories (33.3-43.3%) (Table 4).

Table 4. Total/overall percentage of oysters in which *Bonamia ostreae* was detected using a combination of diagnostic methods by pairs at UCC, CIMA and Cefas. The highest percentage detected at each laboratory is marked with bold characters.

	Heart imprints + Histology	Heart imprints + PCR	PCR + Histology	Heart imprints + ISH	PCR + ISH	Histology + ISH
UCC	33.3% (10/30)	63.3% (19/30)	63.3% (19/30)	46.7% (14/30)	**66.7% (20/30)**	46.7% (14/30)
CIMA	40% (12/30)	50% (15/30)	**53.3% (16/30)**	43.3% (13/30)	**53.3% (16/30)**	43.3% (13/30)
Cefas	43.3% (13/30)	63.3% (19/30)	**66.7% (20/30)**	40% (12/30)	63.3% (19/30)	40% (12/30)

Agreement in positive cases between diagnostic methods in each laboratory

A combination of heart imprints + histology screening had the highest percentage of infected individuals in common in UCC (90%) and CIMA (83.3%) while a combination of histology + ISH had the highest percentage in Cefas (83.3%). Histology + PCR at UCC had the lowest agreement (21.1%). In CIMA, PCR + ISH had the lowest agreement (44%) while in Cefas, heart imprints + PCR had the lowest agreement, 42.1% (Table 5).

Table 5. A comparison of the agreement between methods for *B. ostreae*-positive (%) oysters at each laboratory.

	Heart imprints			PCR			Histology			ISH		
	UCC	CIMA	Cefas	UCC	CIMA	Cefas	UCC	CIMA	Cefas	UCC	CIMA	Cefas
Heart imprints				26.3% (5/19)	73.3% (11/15)	42.1% (8/19)	90% (9/10)	83.3% (10/12)	61.5% (8/13)	57.1% (8/14)	46.2% (6/13)	58.3% (7/12)
PCR	26.3% (5/19)	73.3% (11/15)	42.1% (8/19)				21.1% (4/19)	63% (10/16)	50% (10/20)	30% (6/20)	44% (7/16)	47.4% (9/19)
Histology	90% (9/10)	83.3% (10/12)	61.5% (8/13)	21.1% (4/19)	63% (10/16)	50% (10/20)				58.3% (7/12)	46.2% (6/13)	83.3% (10/12)
ISH	57.1% (8/14)	46.2% (6/13)	58.3% (7/12)	30% (6/20)	44% (7/16)	47.4% (9/19)	58.3% (7/12)	46.2% (6/13)	83.3% (10/12)			

Discussion

In the samples screened, the overall prevalence of infection was approximately 33%. Most consistency occurred amongst laboratories with heart imprints and the most variability with PCR. To assess clinical infections, heart imprints and histology gave good reproducibility amongst the laboratories indicating that, apart from very low levels of infection (i.e. where just a few *Bonamia ostreae* cells are present), screening using these methods gave fairly consistent results. Light infections may have been overlooked due to the small size and focal nature of *B. ostreae* and the subjective nature of microscopic methods, wherein just an imprint or a single transverse section, several microns thick, is screened (Culloty et al. 2003, da Silva & Villalba 2004, Lynch et al. 2008). Also the overall success of these methods is highly dependent on the fixation of the tissue and the preparation of the slides, thus these ungovernable technical issues may have occurred with some of the slides (Diggles et al. 2003, Lynch et al. 2008).

Overall, PCR gave the highest rate of positive detection for *B. ostreae* DNA in all three laboratories but also had the lowest agreement amongst laboratories. This was to be expected as many studies have found that PCR produces a higher number of positives than light microscopy (Diggles et al. 2003, Lynch et al. 2005, Balseiro et al. 2006, Culloty & Mulcahy 2007, Lynch et al. 2008). Light *B. ostreae* infections might give variable results amongst laboratories though. This might be particularly true in this study, where different individual pieces of tissue were screened at each laboratory for PCR. Low infection levels and low numbers of *B. ostreae* cells, localised in particular host cells or tissues, might give variable results depending on whether infected or uninfected pieces of tissue were screened.

The high rate of detection associated with PCR in this study does not necessarily infer that PCR is the most effective detection method. Due to the design of the procedure, the pathogen cannot be visualised thus a viable pathogen and/or infection may not be present. The PCR may be detecting traces of *B. ostreae* DNA (Burreson 2000). In contrast, since gill tissue was used in this study, PCR may also be detecting early infections, which have not yet become systemic.

At present, the OIE recommends that histology or tissue imprints are used for surveillance and when mortalities occur, PCR can be used in addition to these techniques (OIE 2012). Overall the findings of this study would indicate that at least two methods should be used at all times for the accurate detection of prevalence of infection within a sample - a microscopy based technique for visualisation of the parasite, the infection and associated pathology, and a molecular based technique. da Silva & Villalba (2004) concluded that every technique gives rise to false negatives, thus using several methods would lower the risk of misdiagnosing each individual. Lynch et al (2008) found the use of any two methods increased the likelihood of detecting *B. ostreae.* Of the traditional microscopic methods, heart imprints appear to be the best choice as it had the highest agreement of infected individuals amongst the three laboratories. It was also the most effective method at detecting light infections. Heart imprints are also fast to prepare (Diggles et al. 2003), an important factor when screening animals prior to movement. PCR may be considered the best molecular method of detection because although ISH has the ability to visualise the parasite, detection of light infections was greater with PCR. Also factoring in other aspects such as cost and preparation time, ISH is expensive and time consuming to undertake (Diggles et al. 2003, Lynch et al. 2008).

Diggles et al. (2003) recommended the refinement of heart imprints, using a method similar to the dot–blot methods used by Lightner & Redman (1998), in which haemolymph is hybridised with a digoxigenin-labelled probe. The combination of a quick and easy traditional method with a modern molecular technique may possibly lead to a sole, reliable diagnostic test for *B. ostreae.*

The variation, in results, amongst the three laboratories in the methods PCR and ISH, both complex and lengthy molecular tests with the possibility for highly varied methodologies, may be due to the different protocols used by each laboratory for both methods. Heart imprints and histology, both straightforward and concise methods, showed very little variation amongst the laboratories. These findings may imply the need for a standardised, fixed protocol for each molecular based method to allow for optimisation of each assay and consistency in findings throughout all laboratories in the screening for *B. ostreae.* The fact that the gill pieces used for PCR diagnosis were not the same for the three laboratories, could also contribute to increase variability of results with this method, specially in light infections.

Though the EU strictly regulates the movement of *Ostrea edulis*, the fact that no diagnostic method is 100% accurate is of great concern. This study indicates that, at present, the use of both a microscopy based method, to allow for visualisation of the parasite, and a molecular method, to increase sensitivity in low infections, would allow for a more precise diagnosis of *B. ostreae* and that particular caution is required when screening light infections.

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Chapter 3

Part A

Assessment of the impact of a pathogen, *Bonamia ostreae*, on *Ostrea edulis* oyster stocks with different histories of exposure to the parasite in Ireland.

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Assessment of the impact of a pathogen, *Bonamia ostreae*, on *Ostrea edulis* oyster stocks with different histories of exposure to the parasite in Ireland

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Abstract

The protozoan parasite *Bonamia ostreae* has decimated *Ostrea edulis* stocks throughout Europe over the past four decades. A study of two stocks of *O. edulis* in Ireland with varying periods of exposure to *B. ostreae*, 5 years and 22 years, was undertaken. The objective of the study was to determine if varying lengths of exposure would translate into observations of differing susceptibility to *B. ostreae.* A number of oyster beds within each area were screened. The study was carried out over 13 months to investigate seasonality and the role of environmental parameters, population density and size on disease development. Of particular interest was the fact that prevalence of infection in both stocks was very similar. The stock that had been exposed for 22 years had a similar prevalence, intensity and seasonality of infection as the stock infected for 5 years.

B. ostreae was detected in both stocks throughout the year with the highest prevalence in spring, possibly related to the increase in water temperature and/or oysters directing their energy towards gametogenesis. The study indicated that oyster stocks can maintain themselves over extended periods of time in *B. ostreae* endemic areas. However, prevalence of *B. ostreae* will remain relatively stable within the stock without some intervention to improve resistance levels e.g. by breeding for resistance over a number of years. Some natural resistance to infection will build up in individual oysters but in natural populations this will continually be diluted by cross fertilisation with more susceptible oysters.

Introduction

The European flat oyster's (*Ostrea edulis*) natural range extends from the coast of Norway, south to Morocco and into the Mediterranean basin as far as the Black Sea coast (FAO 2010). Due to overfishing, habitat destruction and exotic diseases (Culloty & Mulcahy 2007), wild populations are now scarce throughout Europe with disease free, natural beds, only occurring in isolated regions along the west coasts of Ireland, Scotland, Scandinavia and in the Limfjord region of Denmark.

Over the last forty years, European aquaculture production of *O. edulis* has shown a drastic decline from a peak output of nearly 30,000 tons in 1961 to 6,000 tons today, mainly due to the impact of two parasitic diseases, *Marteilia refringens* and *Bonamia ostreae* (Lallias et al. 2009). With ongoing infection of *B. ostreae* in populations and a considerable shift to *Crassostrea gigas* farming, the industry has been unable to recover to production figures achieved in the 1960's, despite numerous efforts including prohibiting relaying of oysters, fallowing beds and cleansing sites (Hugh-Jones 1994, Culloty & Mulcahy 2007, FAO 2010). Ireland, as with the rest of Europe, suffered a dramatic decline in the species, even before the introduction of parasitic diseases, possibly due to overexploitation and poor management of fisheries (Culloty & Mulcahy 2007).

One of the major reasons for the decline of *O. edulis* is *B. ostreae*, an intracellular haplosporidian parasite found in flat oysters in Europe (Balouet et al. 1983, McArdle et al. 1991, van Banning 1991), and on the Atlantic and Pacific coasts of the USA (Elston et al. 1986, Friedman et al. 1989, Friedman & Perkins 1994). The parasite was first described in Europe in Brittany, France, by Pichot et al. (1979). *B. ostreae* was first detected in *O. edulis* in Cork harbour, on the south coast of Ireland in 1987 (McArdle et al. 1991). A report on the presence of *B. ostreae* in a consignment of

oysters from Clew Bay on the west coast, exported to France was made in 1988. Later screening of 1,500 Clew Bay *O. edulis* in 1991 failed to confirm the presence of *B. ostreae* (McArdle et al. 1991). Presence of the parasite in the bay was finally confirmed in 1994, but infections occurred at a very low prevalence (Marine Institute 2006). On the north coast of Ireland, Lough Foyle was the stock in which *B. ostreae* was most recently detected, in Spring 2005, with 43% prevalence of infection in one of three beds screened (Loughs Agency 2011).

A range of epidemiological studies on *B. ostreae* and its host have indicated differences in seasonality in this disease with size, age and environmental conditions, all impacting on prevalence and intensity of infection observed at different sites (Cáceres-Martínez et al. 1995, Culloty & Mulcahy 1996, Engelsma et al. 2010). Long term epidemiological studies of other oyster species indicate that some changes in host parasite dynamics (the nature of which have not always been elucidated) can occur over an extended period of time, with changes in host susceptibility, phenotypic and genotypic variation in the host and parasite and changes in environmental conditions all contributing to different disease patterns being observed in populations (Carnegie & Burreson 2012).

The aim of this study was to investigate whether exposure to *B. ostreae* over varying periods of time (5 and 22 years) in wild, natural oyster stocks would result in changes in the host-parasite dynamics. It was considered that this reduction in host susceptibility might occur following exposure over an extended period of time, thereby affecting prevalence and intensity of infection and thus subsequent impacts on stocks. The role of environmental parameters, population density and size classes for host-parasite relationships were also assessed, when information was available. To achieve these objectives, two stocks – Clew Bay with one of the earliest records

of the disease in Ireland and Lough Foyle, the stock with the most recent detection of the parasite, were chosen to monitor infection over a 13-month period, encompassing spatial and seasonal variations.

Materials And Methods

Study areas

Two stocks in Ireland were selected; Clew Bay, a wild fishery that has been affected by *Bonamia ostreae* since 1988, situated on the west coast and Lough Foyle, a wild fishery where *B. ostreae* was first detected in 2005, found on the north coast of Ireland (Fig. 1).

Clew Bay (53.8333° N, 9.8000° W) is a westerly facing bay in Co. Mayo on the West Coast of Ireland and is dominated by a drowned drumlin field (Fig. 1). The bay covers an area of approximately 16000 ha. The inner bay is shallow with an average depth of 10m increasing seawards to 20m. The seabed is a mixture of sand, gravel, mud and boulders. The tidal range is 5 metres. Fourteen rivers flow into the bay. It has been categorised as a class A area under Directive 91/492/EEC - Classification of Shellfish Production Areas (http://sfpa-ie.access.secure-ssl-servers.biz/index.php?q=shellfish). The bay is characterised by the presence of 117 islands and there are numerous oyster beds seeded from natural settlement.

Lough Foyle (55.1167° N, 7.0833° W) is a northerly facing bay bordering Co. Donegal and Co. Derry on the north coast of Ireland (Fig. 1). The Lough covers an area of approximately 18,600 ha. The average depth of the Lough is 5m, reaching a max of 15m. The seabed is a mixture of poorly sorted sands, gravels and mudflats. The tidal range is 3 metres. Three rivers flow into the Lough. It has been categorised as a class B area under Directive 91/492/EEC - Classification of Shellfish Production Areas. There are a total of 16 productive native oyster beds seeded from natural settlement in the bay, covering an area of 7081 ha.



Fig. 1. Location and map of the two selected sampling sites, Clew Bay and Lough Foyle, in Ireland.

Sampling

Samples of live *Ostrea edulis* were collected from the two stocks, Clew Bay and Lough Foyle, at three monthly intervals, beginning October 2010 and finishing in October 2011 (October 2010, January 2011, April 2011, July 2011, October 2011). The samples were taken from seven oyster beds within Clew Bay (Fig. 1, Table 1) and five oyster beds within Lough Foyle (Fig 1, Table 2). Three of the beds sampled in Lough Foyle, Site A, B and C, all form part of a larger bed termed Southside. 8-60 oysters were collected on each bed on each sampling date. The sample size depended on the availability of oysters on the bed with a desired sample size being 60 individuals per bed. In total 2,475 oysters were collected and examined over the 13 month period – 1,097 from Clew Bay and 1,378 from Lough Foyle (Table 3). Oysters were processed immediately upon arrival at the laboratory with the whole weight (g) and length (cm) of each oyster being recorded before oysters were opened.

Table 1. Name and location of sampling beds in Clew Bay.

Site no.	Name of bed	Grid reference	
1	Rosgibbilean	53°53'22.10"N	9°35'47.00"W
2	Rosbeg	53°51'24.80"N	9°34'54.00"W
3	Rosbarnagh	53°52'29.80"N	9°35'34.40"W
4	Newport River	53°52'56.90"N	9°35'38.80"W
5	Friar's Island	53°53'8.10"N	9°37'48.00"W
6	Inishloy North	53°51'53.00"N	9°35'14.08"W
7	Inishloy South	53°51'39.10"N	9°35'15.10"W

Table 2. Name and location of sampling beds in Lough Foyle.

Site no.	Name of bed	Grid reference	
1	Sandy Ridge	55° 8'0.20"N	7° 4'5.94"W
2	Flat ground	55° 5'6.82"N	7° 6'8.55"W
3	Site A	55° 6'9.88"N	7° 4'3.42"W
4	Site B	55° 6'7.62"N	7° 4'3.77"W
5	Site C	55° 7'1.69"N	7° 4'3.07"W

Table 3. Number of oysters screened from the two stocks on each sampling date over the study period.

	Clew Bay							**Lough Foyle**				
	Rosbeg	Rosgibbilean	Rosbarnagh	Newport River	Friar's Island	Inishloy North	Inishloy South	Sandy Ridge	Flat Ground	Site A	Site B	Site C
Oct-10	45	14	19	31	9	47	28	42	46	60	60	60
Jan-11	35	31	39	30	24	32	26	45	50	60	60	60
Apr-11	44	30	40	30	20	30	31	40	56	58	53	44
Jul-11	35	32	37	48	26	33	32	50	60	60	60	59
Oct-11	33	33	30	34	24	32	33	56	60	60	59	60
Total	**192**	**140**	**165**	**173**	**103**	**174**	**150**	**233**	**272**	**298**	**292**	**283**

Cytological processing and examination

Diagnosis of *Bonamia ostreae* infection was carried out using ventricular heart imprints. The imprints were air dried before being fixed in methanol for two minutes and stained with Hemacolour 2 and 3 (Merck) (Culloty et al. 1999) and washed prior to mounting in DPX. The imprints were examined under light microscopy at 400x magnifications. The intensity of *B. ostreae* infection was determined using the following scale:

Class 0: No *B. ostreae* cells observed.

Class 1: 1–10 *B. ostreae* cells observed.

Class 2: 11–100 *B. ostreae* cells observed.

Class 3: *B. ostreae* cells observed in all fields of vision

Class 4: *B. ostreae* cells observed in all cells (Bachère et al. 1982, Culloty et al. 2004).

DNA isolation and Polymerase chain reaction analysis

A small sample of gill was collected from each animal and frozen at -20°C for DNA based diagnosis. In cases where heart imprints could not be obtained or could not be subsequently read, PCR analysis was used to confirm *Bonamia ostreae* DNA presence. DNA was extracted from the tissue using the Chelex-100 method (Walsh et al. 1991, Lynch et al. 2008) and stored at –20°C. PCR analyses were performed with primers Bo Boas, which amplified 300 bp from the SSU rRNA gene, using the method of Cochennec et al. (2000). All PCR assays included positive controls (*B. ostreae* DNA) and a negative control (double distilled water). The PCR products

were run on 2% agarose gel in TE buffer gel (110V and for 40 mins) stained with ethidium bromide (10mg/L).

Environmental Data

Temperature and salinity data was collected by Bord Iascaigh Mhara (BIM) for Clew Bay and by the Loughs Agency for Lough Foyle (www.loughs-agency.org). Star Oddi loggers, deployed at several intertidal sites in Clew Bay north, acquired Clew Bay temperature and salinity data. The loggers recorded on the hour every hour. Electronic instruments that recorded every 15 minutes acquired Lough Foyles' temperature and salinity data. Several technical issues occurred during the gathering of data in Clew Bay so environmental data was compiled from information from several loggers in the north of the bay. In Lough Foyle, as a result of technical difficulties, no environmental data were available from July 2011 to October 2011.

Statistical analysis

The data were analysed with the software SPSS12.0.1 (PASW Statistics 17 statistical software for Microsoft Windows). Logistic regressions were used to determine if there was a significant difference between the prevalence of infection in Clew Bay and Lough Foyle each sampling month and to determine if there was a significant difference between each sampling month, if it was concluded that there was no significant difference between the prevalence of infection in both stocks. A Shapiro-Wilk normality test was used to check the normality of the data and to determine if there was a correlation between prevalence of infection and weight and length.

Results

Prevalence of infection on oyster beds sampled at each site

Clew Bay

Of the 1,097 oysters screened from Clew Bay over the 13 months of the trial, 10% were infected. The October 2010 results are based on PCR screening and all other months were based on screening of heart imprints. All oysters diagnosed as negative by imprints were subsequently screened using PCR. Infection was found in the oysters all year round. Of the five months sampled over the study period, oysters screened in April 2011 had the highest mean prevalence of infection at 18.7% ± 10.3 while the lowest mean prevalence was recorded in January 2011 (6% ± 4.6) (Fig 2a). A preliminary study of oyster densities in Clew Bay has indicated that current densities for the area are <1 oyster per m^2 (M. Hannan, BIM, *pers comm*.).

Prevalence of infection varied from 6% in January 2011 to 18.7% in April 2011. When the various beds were compared in Clew Bay over the study period, mean prevalence of infection was highest in the Rosbeg bed (15.6% ± 9.6, Fig. 3a) and lowest in Newport River (1.7% ± 1.5, Fig. 3a). The bed that demonstrated the highest prevalence of infection in any one sample was Rosgibbilean in October 2010 (28.6%). Some beds had infected oysters on all sampling dates with some variations in prevalence over the study period: Rosbeg, 7 - 31%, Rosgibbilean, 3 - 28.6% and Rosbarnagh, 3.3 - 21.6%. Four beds had oysters with low or no infection in certain months over the study period: Newport River, 0 - 3.3%; Friars Island, 0 - 11.5%; Inishloy North, 0 - 26.7% and Inishloy South, 0 - 11.5%.

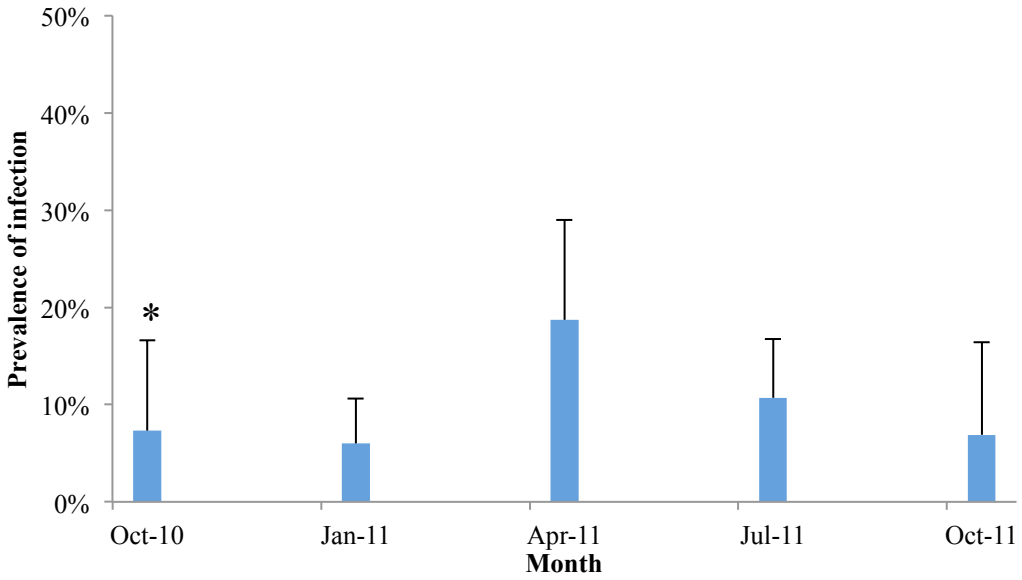
Lough Foyle

Of the 1,378 oysters screened from Lough Foyle, 11% were infected with *Bonamia ostreae.* As with Clew Bay, infection was found all year round in Lough Foyle. Of the five months sampled over the study period, oysters screened in April 2011 had the highest mean prevalence of infection at 21.5% ± 17.8, while the lowest mean prevalence recorded was in July 2011 (5.5% ± 4.3) (Fig 2b). A comprehensive study of *Ostrea edulis* density was carried out in Lough Foyle in spring 2011 by the Loughs Agency (Loughs Agency 2012). Overall, population density for the Lough was 1.04 oysters per $m^{2,}$ though the population of each bed varied greatly. Site A, located on the bottom of the Southside bed, has a population density of 0.326 oysters per m^2. Site C (15.2% ± 15.7) located at the top of Southside bed had a density of 0.26 oysters per m^2; Sandy ridge had 0.034 oysters per m^2 and Flat ground 0.09 oysters per m^2.

When the various beds were compared in Lough Foyle over the study period, mean prevalence of infection was highest in the Southside Site A bed (16.4% ± 11, Fig. 3b) and lowest in Flat ground (0.4% ± 0.8, Fig. 3b). The bed that demonstrated the highest prevalence of infection in any one sample was Southside Site C in April 2011 (43.2%). As with Clew Bay, some beds had infected oysters on all sampling dates, with some variations in prevalence over the study period: Sandy Ridge, 3.6 - 24%; Southside Site A, 7 - 31%; Southside Site B, 8.3 - 24.5% and Southside Site C, 5.1 - 43.2%. Only one bed had oysters with very low or no infection over the study period - Flat ground, 0 - 2%. The bed with oysters with the highest prevalence of infection was Southside Site A (16.4% ± 11), the bed with the highest population density in Lough Foyle.

There was no significant difference in the prevalence of infection observed in Clew Bay and Lough Foyle in the months studied (Logistic regression, P=0.343). As there was no significant difference between the two stocks, statistically they may now be treated as one sample thus the prevalence of infection of each sampling month overall was analysed for significance. Statistically significant differences were found between October 2010 and October 2011 (P=0.004), and April 2011 and October 2011 (P=0.001).

(a)



(b)

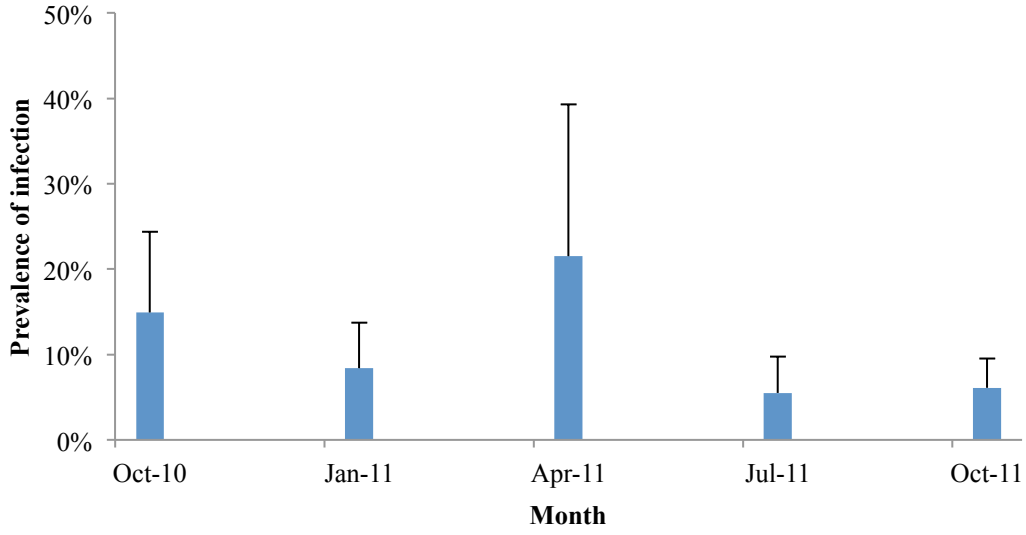
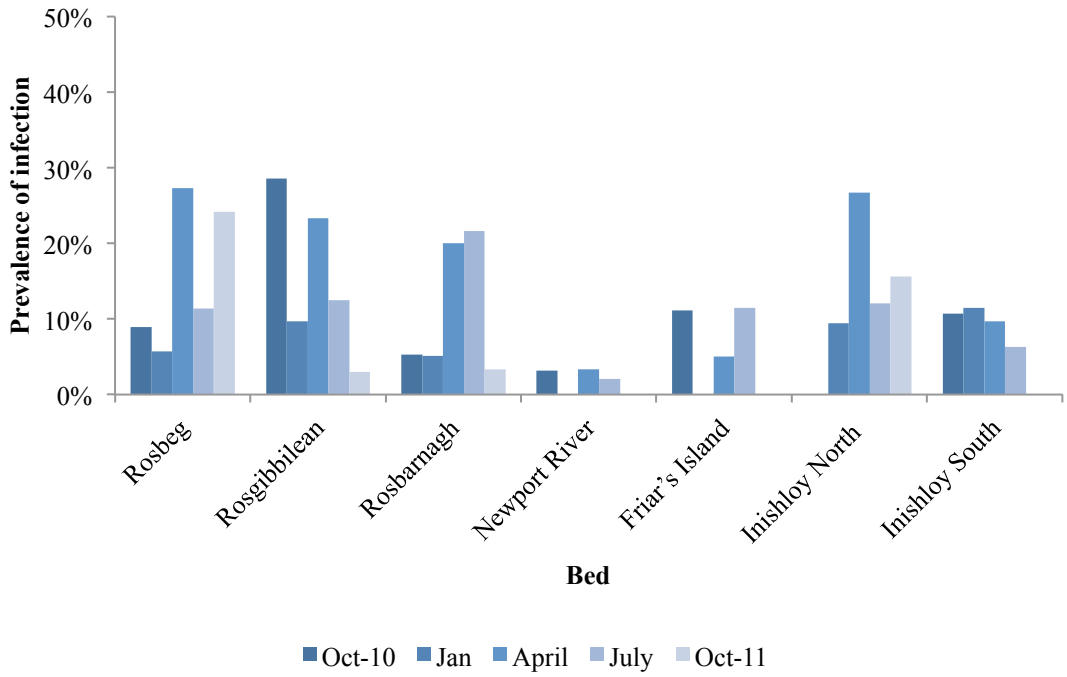


Fig. 2. Overall prevalence (%) of *Bonamia ostreae* infection in (a) Clew Bay and (b) Lough Foyle. Mean value ± SD.

*Prevalence of infection determined using PCR

(a)



(b)

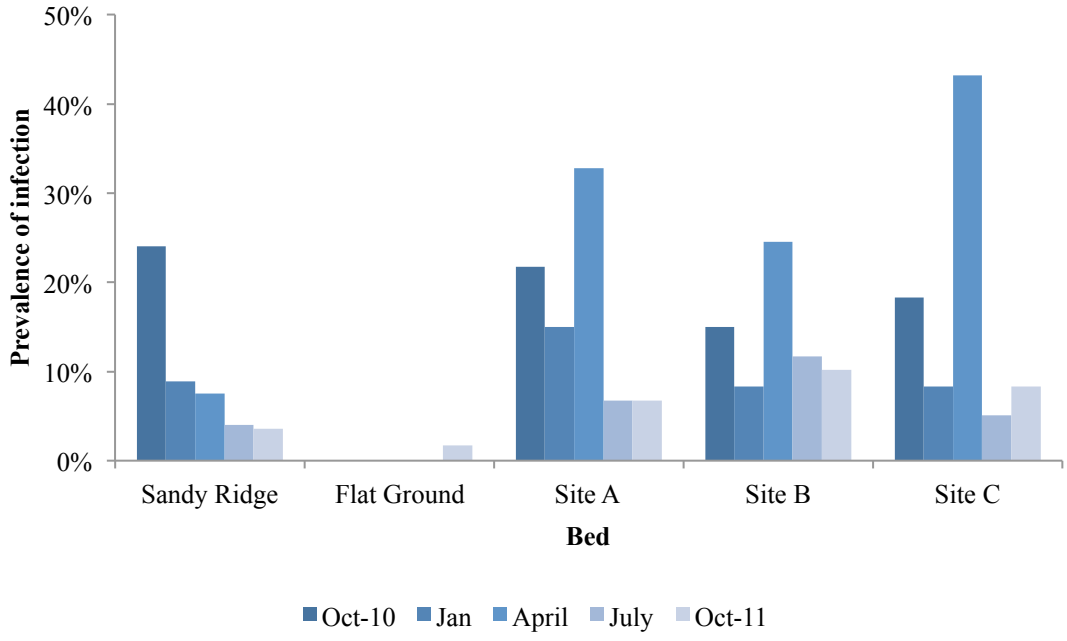


Fig. 3. Prevalence (%) of *Bonamia ostreae* in screened oysters from different beds within (a) Clew Bay and (b) Lough Foyle over the study period

Intensity of infection in both stocks and within each oyster bed

Clew Bay

Heart imprints taken from Clew Bay oysters in October 2010 could not be read due to fixation problems, thus intensity of infection could not be determined for that month. Of the other months screened, the percentage of uninfected oysters (i.e. class 0) varied from 80.9% (April 2011) to 94% (January 2011). Of the infected oysters, the greatest number of class 1 infections was observed in January 2011 (1.4%). The greatest number of class 2 infections was observed in April 2011 (11.6%), class 3 in April 2011 (8%) and class 4 in July 2011 (0.8%) (Fig. 4a). Of the infected oysters in Clew Bay screened over the study period, class 2 was the most frequently observed intensity in four out of the seven beds – Rosbarnagh (50%), Newport River (100%), Friar's Island (60%) and Inishloy North (65%) (Fig.5). Class 3 was the most frequently observed intensity in the remaining three beds – Rosbeg (52%), Rosgibbilean (50%) and Inishloy South (37.5%). Class 4 infections were only found in three beds - Rosgibbilean (6.3%), Rosbarnagh (10%) and Inishloy North (5%).

Lough Foyle

In Lough Foyle, the percentage of uninfected oysters varied from 78.5% (April 2011) to 95% (October 2011). Of the infected oysters, the greatest number of class 1 infections was observed in October 2011 (4.3%). The greatest number of class 2 infections was observed in April 2011 (9.6%), class 3 in April 2011 (10.4%) and class 4 in October 2010 (1.6%) (Fig.4b). Of the infected oysters in Lough Foyle, class 3 was the most frequently observed intensity, in two out of the five beds – Flat ground (100%) and Southside Site C (44.4%) (Fig.6). Both class 2 and 3 intensities were most frequently observed in two beds – Sandy ridge (38.1% each) and

Southside Site B (43.5% each). Class 2 was the most frequently observed intensity at Southside Site A (47.2%). Class 4 infections were only found in two beds – Sandy ridge (9.5%) and Southside Site B (7.5%). All but Flat ground had class 1 infections – Sandy ridge (14.3%), Southside Site A (8.3%), Southside Site B (7.5%) and Southside Site C (13.9%).

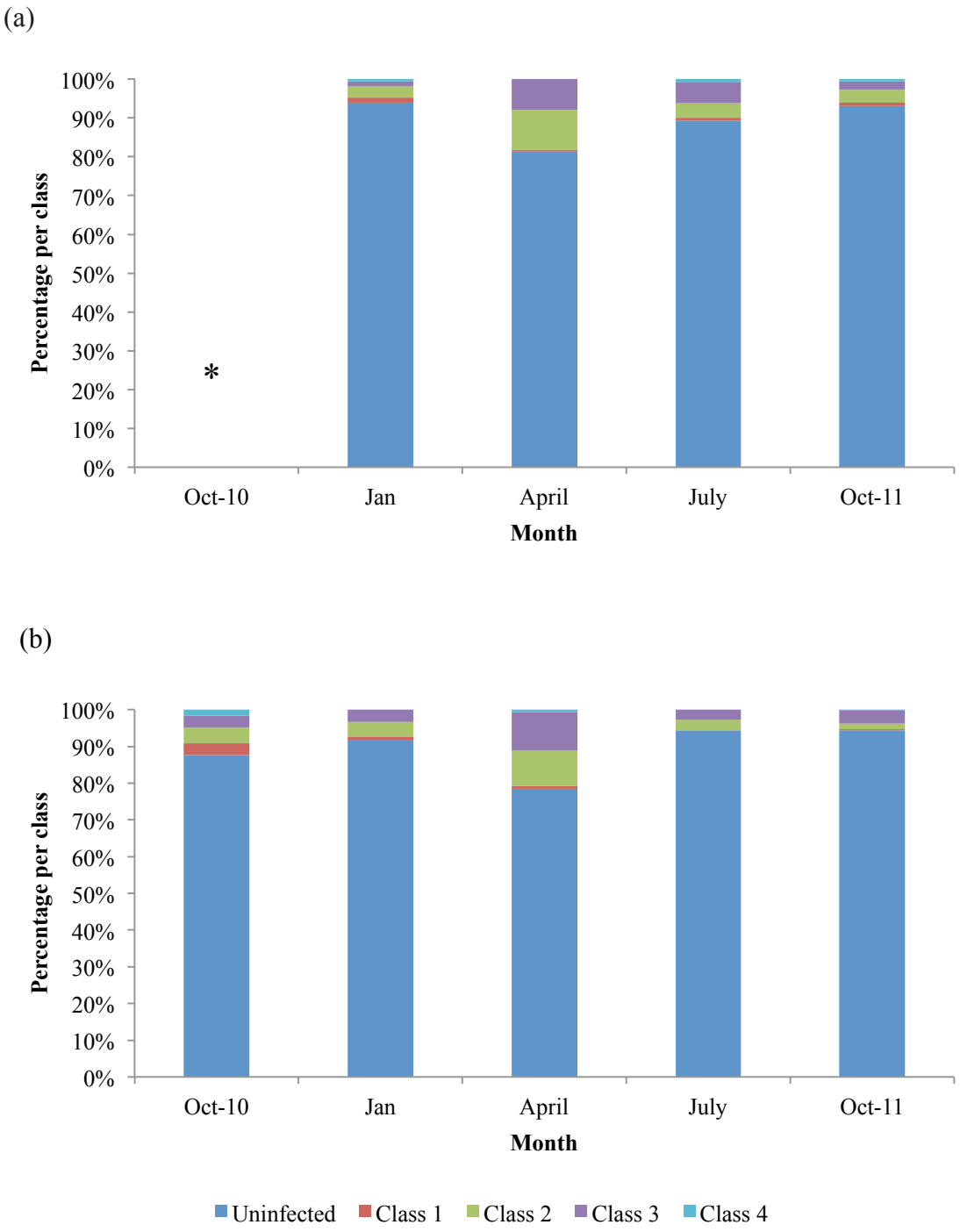
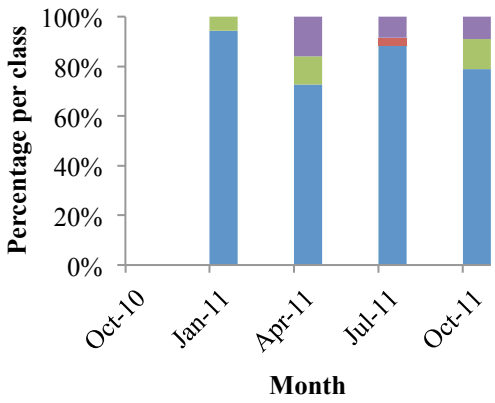


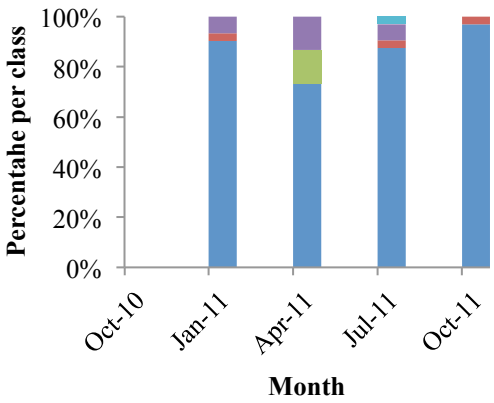
Fig. 4. Overall variation in intensity of infection (%) observed in (a) Clew Bay and (b) Lough Foyle over the study period from October 2010 to October 2011.

* No results due to unavailability of heart imprints

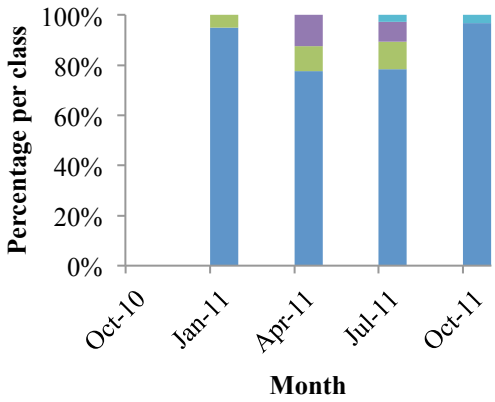
(a)


Percentage per class
100%
80%
60%
40%
20%
0%
Oct-10
Jan-11
Apr-11
Jul-11
Oct-11
Month

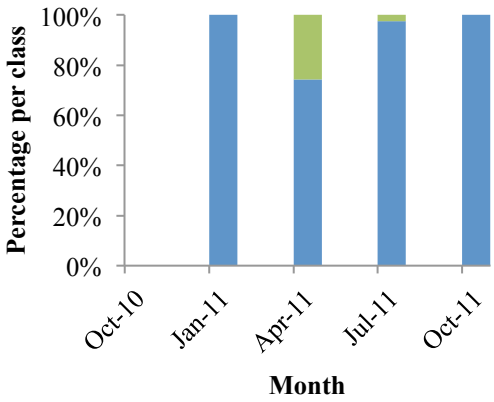
(b)


Percentahe per class
100%
80%
60%
40%
20%
0%
Oct-10
Jan-11
Apr-11
Jul-11
Oct-11
Month

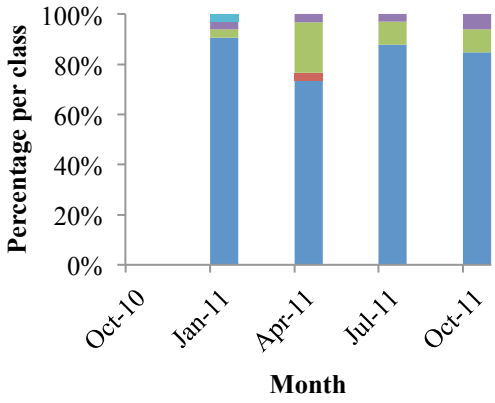
(c)


Percentage per class
100%
80%
60%
40%
20%
0%
Oct-10
Jan-11
Apr-11
Jul-11
Oct-11
Month

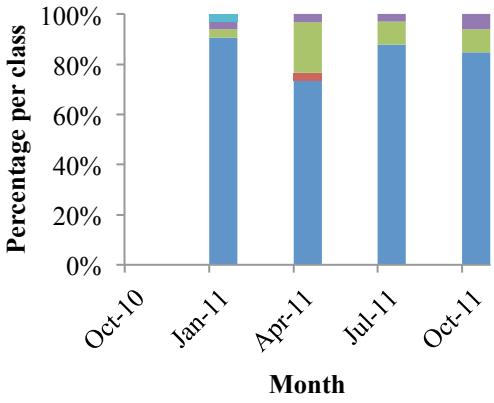
(d)


Percentage per class
100%
80%
60%
40%
20%
0%
Oct-10
Jan-11
Apr-11
Jul-11
Oct-11
Month

(e)


Percentage per class
100%
80%
60%
40%
20%
0%
Oct-10
Jan-11
Apr-11
Jul-11
Oct-11
Month

(f)


Percentage per class
100%
80%
60%
40%
20%
0%
Oct-10
Jan-11
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Jul-11
Oct-11
Month

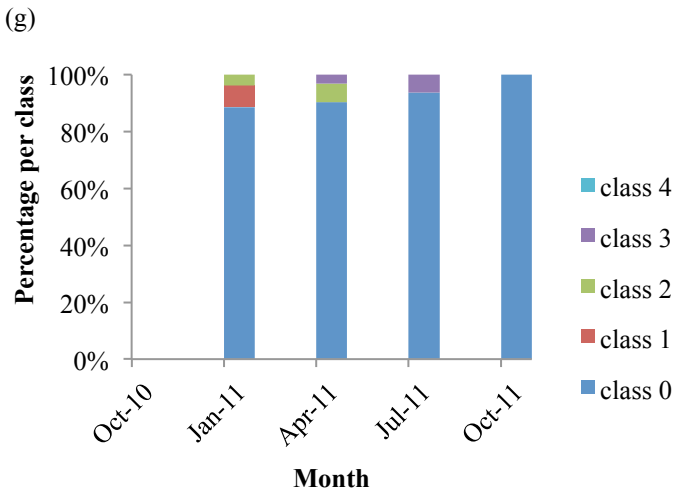


Fig. 5. Intensity of infection (Class 0-4) observed over the sampling period at beds in Clew Bay (a) Rosbeg (b) Rosgibbilean (c) Rosbarnagh (d) Newport River (e) Friar's Island (f) Inishloy North (g) Inishloy South.

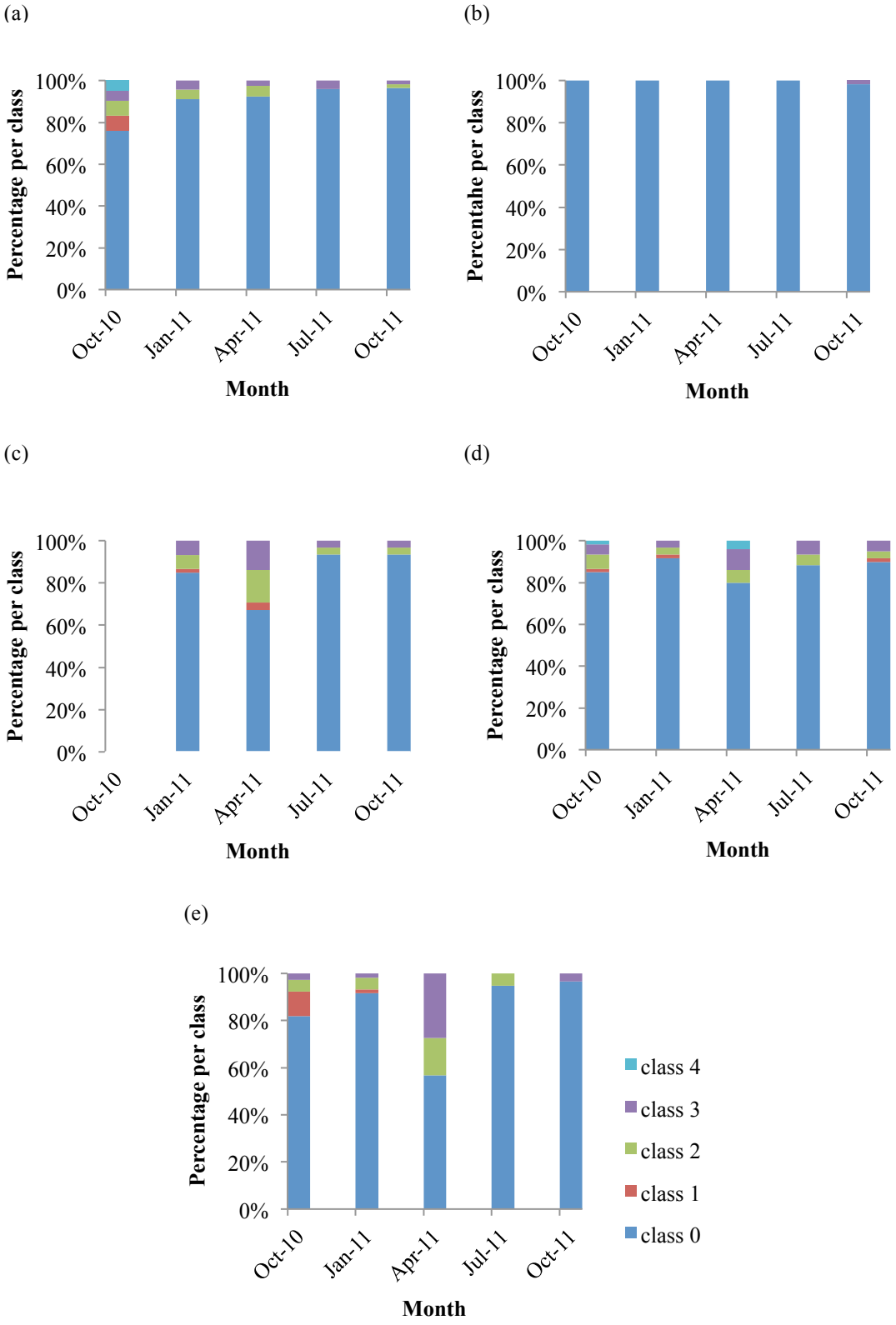


Fig. 6. Intensity of infection (Class 0 - 4) observed over the sampling period at beds in Lough Foyle (a) Sandy ridge (b) Flat ground (c) Site A (d) Site B (e) Site C.

Relationship between oyster size and infection

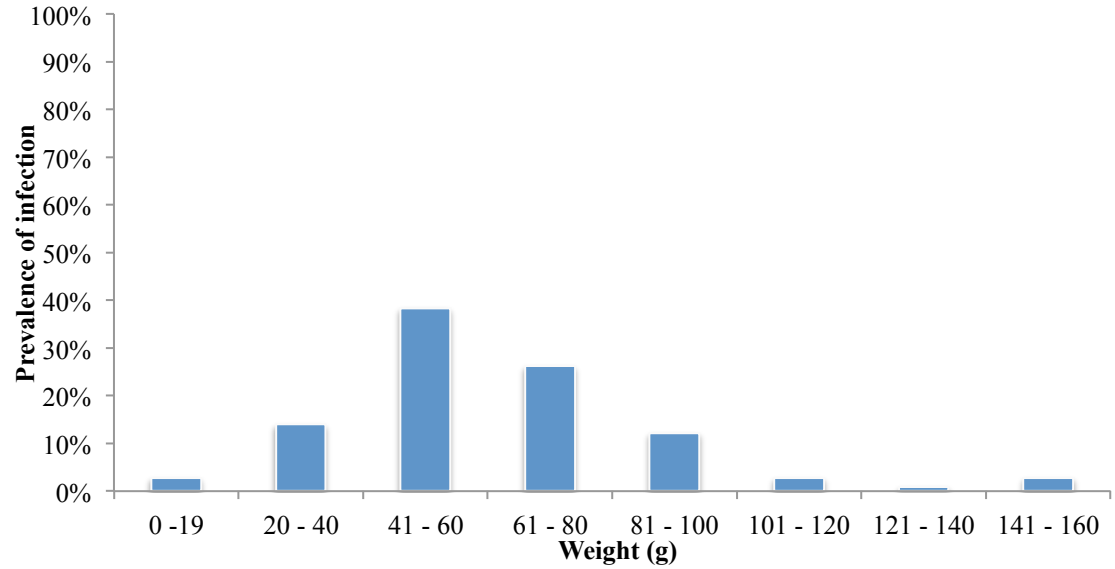
Clew Bay

Collected oysters were divided into weight group of 20 g increments and length group of 10 cm increments. In Clew Bay the weights of oysters sampled varied from 5.4 - 222.1g. The weight group 41 – 60 g had the highest prevalence of infection, 38.3%, followed by 61 - 80 g at 26.2% (Fig. 7a). The lengths of oysters sampled varied from 3.4 – 10.3 cm. Length group 6.1 – 7.0 cm had the greatest prevalence of infection, 39.6%, followed by 7.1 – 8.0 cm at 34.9% (Fig. 8a). There were some large sized oysters present in Clew Bay in terms of both length and weight, indicating they were well past market size. These larger oysters demonstrated a very low prevalence of infection. The rate of infection by length was log normally distributed (P=0.280).

Lough Foyle

The weights of oysters sampled varied from 7.0 - 135.7g in Lough Foyle. Weight group 41 – 60 g had the greatest prevalence of infection, 50.7%, followed by weight group 61 - 80 g at 24% (Fig. 7b). The lengths of oysters sampled varied from 3.6 – 9.0 cm. Length group 6.1 – 7.0 cm had the greatest prevalence of infection, 57.1%, followed by 7.1 – 8.0 cm at 22.7% (Fig. 8b). As with length, the rate of infection by weight was log normally distributed (P=0.846).

(a)



(b)

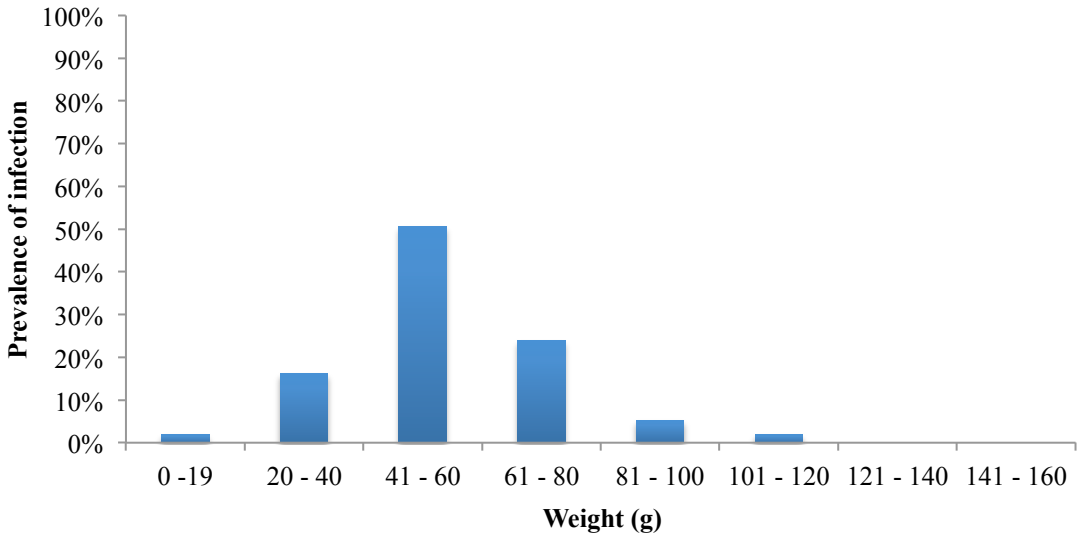
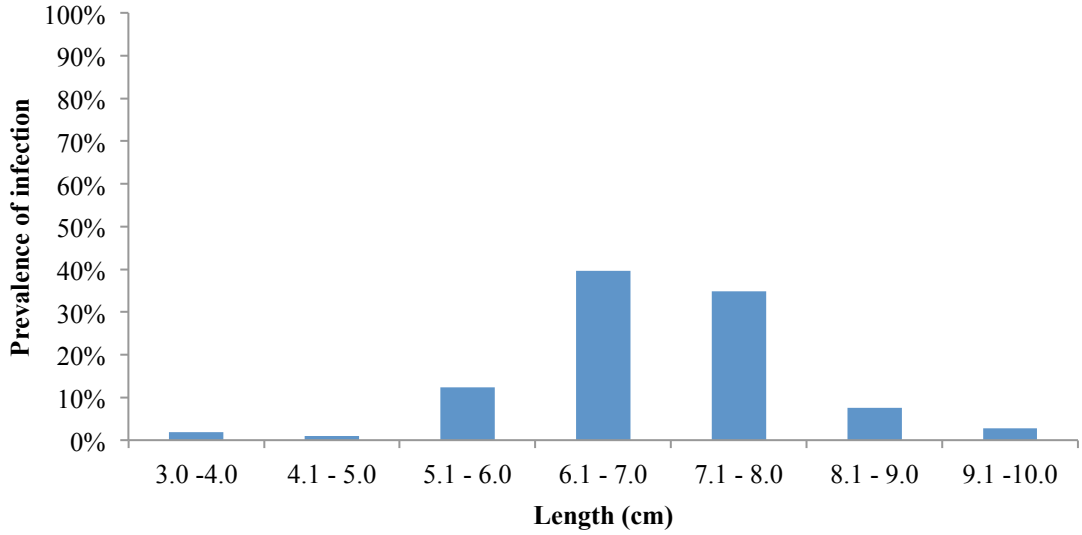


Fig. 7. Prevalence of *Bonamia ostreae* infection (%) in screened oysters of different weight groups in (a) Clew Bay and (b) Lough Foyle.

(a)



(b)

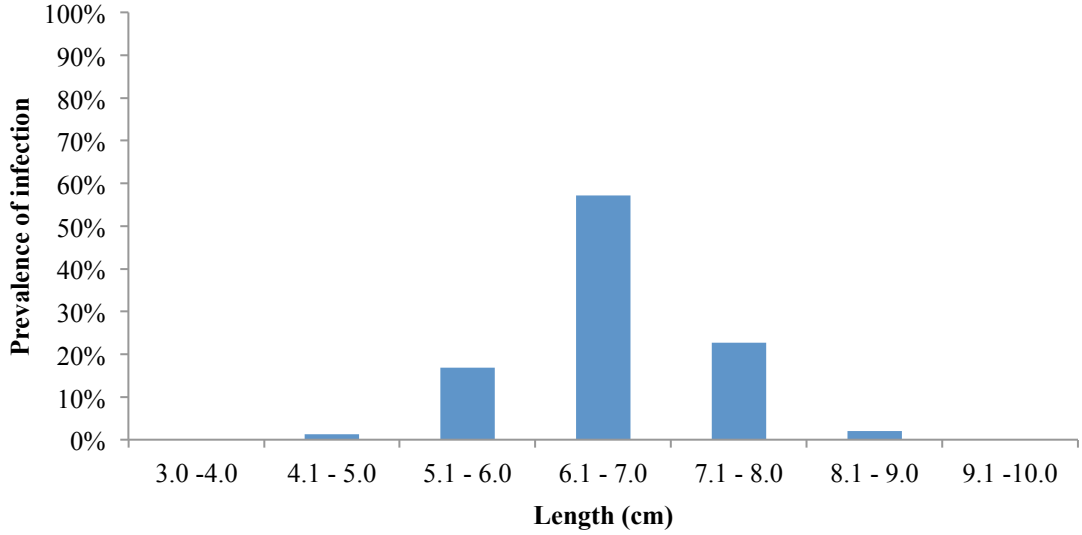
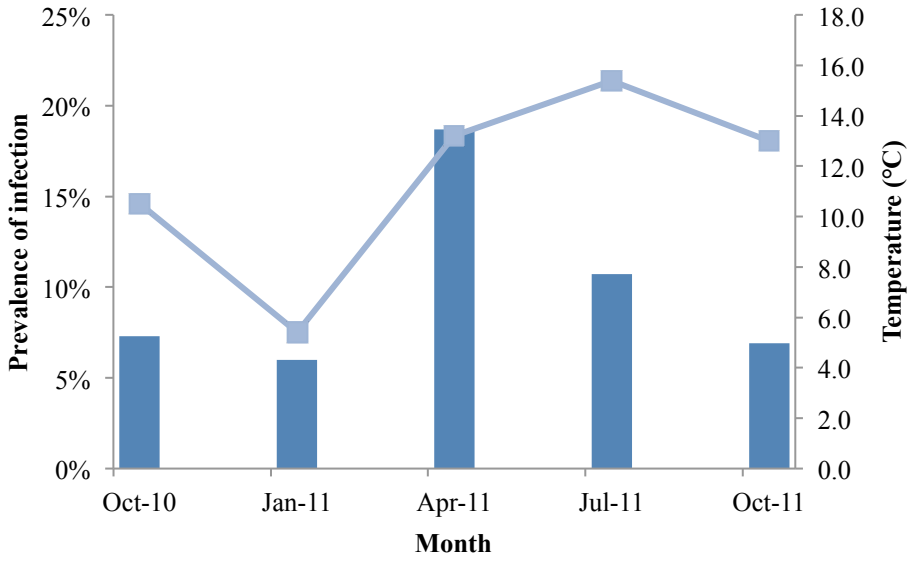


Fig. 8. Prevalence of *Bonamia ostreae* infection (%) in screened oysters of different length groups in (a) Clew Bay and (b) Lough Foyle.

Environmental data

Prevalence of *Bonamia ostreae* infection was greatest in April 2011 at both study sites (18.7% & 21.5% respectively). *B. ostreae* prevalence decreased from April to October 2011, with a dramatic drop from April to July, from 18.7% to 10.7% in Clew Bay and 21.5% to 5.5% in Lough Foyle (Fig. 9). The mean winter salinity levels were much lower in Clew bay compared to Lough Foyle (18.6psu compared to 28.8psu) (Fig. 10). Though overall there was no difference in prevalence of infection in the two stocks, particular oyster beds at both sites demonstrated low or no infection. For example, in Clew Bay, the Newport river bed has been found to have salinity readings as low as 9psu but the highest prevalence of infection observed at that bed was 3.3%. Flat ground in Lough Foyle is also associated with a significant freshwater output. Insufficient data was available to enable statistical analysis on either temperature or salinity.

(a)



(b)

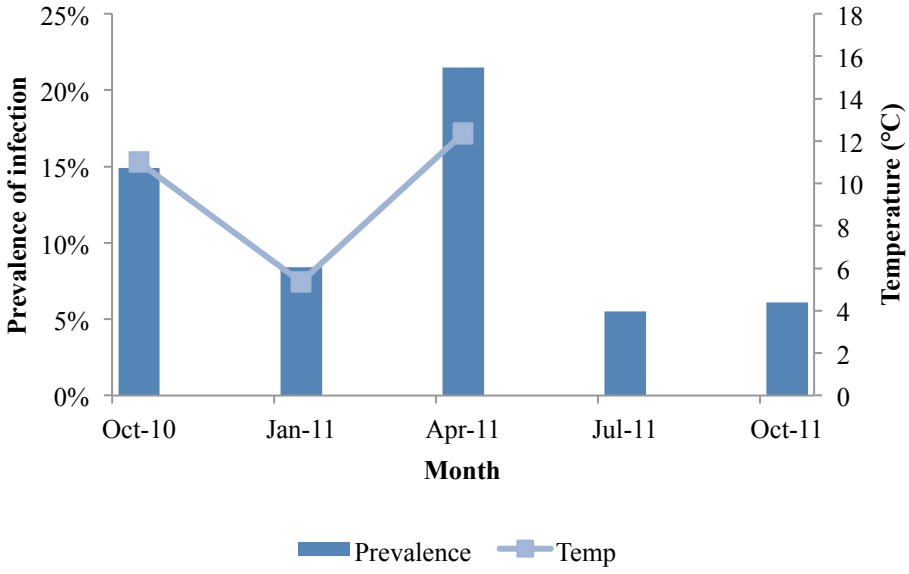
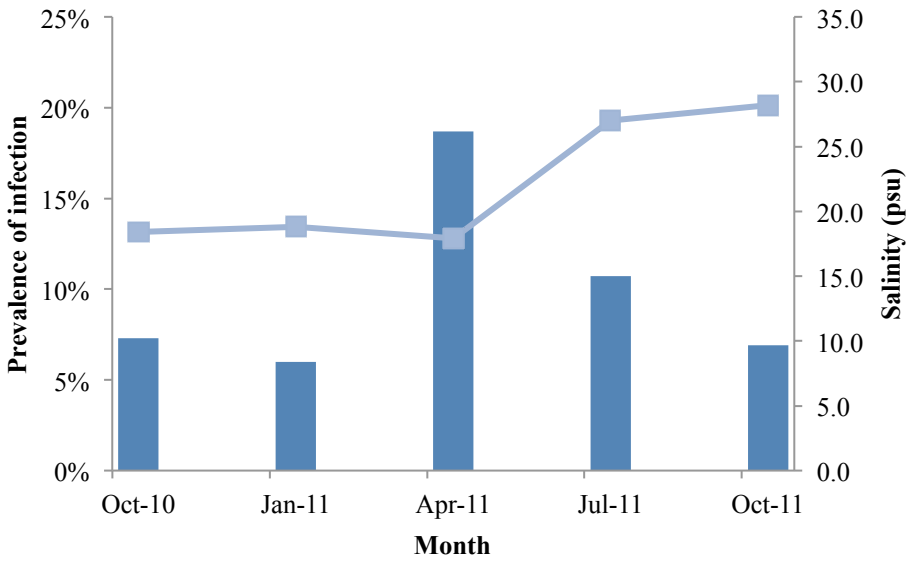


Fig. 9. Relationship between mean prevalence of infection (%) and temperature (°C) at (a) Clew Bay and (b) Lough Foyle over the sampling period.

(a)



(b)

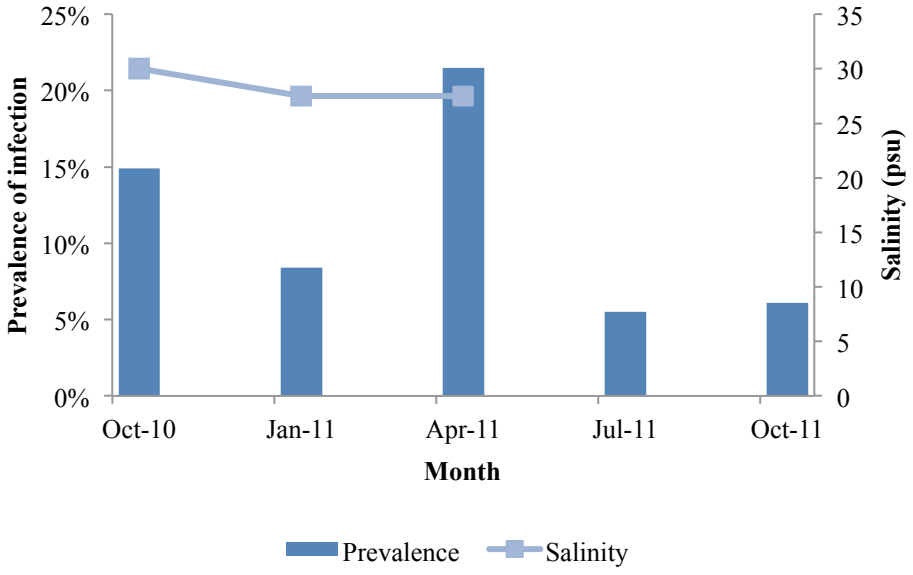


Fig. 10. Relationship between mean prevalence of infection (%) and salinity (psu) in (a) Clew Bay and (b) Lough Foyle over the sampling period.

Discussion

In this study, two Irish oyster stocks with extensive oyster beds and of longstanding economic significance were assessed over 5 seasons, over a 13-month period, to determine the current status of the pathogen *Bonamia ostreae*. The stocks were of particular interest as Clew Bay was one of the first stocks in Ireland in which *B. ostreae* infection was detected, in 1988, and Lough Foyle is the stock in which one of the most recent detections of the disease was made in 2005 (Marine Institute 2006, Loughs Agency 2011). Overall, when all of the oysters screened at each site during the study were combined, the mean prevalence of infection in Lough Foyle was slightly, but not significantly higher than in Clew Bay, 11% compared to 10%. The results demonstrated that stocks such as Clew Bay can survive and maintain themselves even when a pathogen is present in the stock long-term though at relatively low densities. Over this time period, Clew Bay oysters might have developed some resistance and show an overall lower prevalence of infection than the more recently infected Lough Foyle oysters yet there was just a 1% difference. However, when the stocks were examined temporally, it was noted that a greater range of prevalence of infection was observed in Lough Foyle. The development of resistance in *Ostrea edulis* may be possible through natural means over several generations (Elston et al. 1987) or through the controlled management of aquaculture lines by breeding for resistance (Naciri-Graven et al. 1999, Culloty et al. 2001, 2004, Lallias et al. 2008). Other studies have demonstrated that without active intervention e.g. selective breeding, minimal resistance will be observed over a number of generations (Culloty et al. 2004, Engelsma et al. 2010, Lynch et al. *In press*).

Long-term epidemiological data sets are available for other oyster species such as the Eastern oyster *Crassostrea virginica* in the USA, which is infected by two protozoan

parasites, *Haplosporidium nelsoni* and *Perkinsus marinus,* the causative agents of Multinucleated Spore Unknown (MSX) (Andrews 1982) and Dermo disease (Burreson & Ragone Calvo 1996), respectively. *H. nelsoni*, which has been present in *C. virginica* populations since the 1950's, now exists at very low prevalence, due to naturally developed disease tolerance to MSX disease and the possible influence of breeding programs (Carnegie 2009). Using transplantation experiments, Carnegie & Burreson (2011) found that wild *C. virginica* in Chesapeake Bay are developing resistance to *H. nelsoni* through strong selection pressures following exposure to high levels of parasites, resulting in more tolerant oysters that are making a significantly higher contribution to the progeny. In the current study, oysters demonstrated low to medium levels of infection in Clew Bay, indicating that the selection pressure may not be as strong in this stock.

There was a similar pattern in seasonality of infection in both stocks, though their locations relative to each other and the potential stressors are both sites are different – Lough Foyle is relatively sheltered, while Clew Bay is completely exposed to the environment. *Bonamia ostreae* infection occurred all year round in both Clew Bay and Lough Foyle. Similar results have been found throughout the year in other studies from Ireland, France, Spain and the Netherlands (Grizel & Tige 1982, Cáceres-Martínez et al. 1995, Culloty & Mulcahy 1996, Engelsma et al. 2010).

Class 2 and 3 infections were the most observed classes in every bed of the two stocks. The reasons for this may be different at the two sites. Oysters exhibiting class 4 infections were extremely rare as they may have succumbed to infection or have been fished out as they would be market sized, as oysters in the actively fished Lough Foyle were smaller than in Clew Bay. In Clew Bay, with low densities and less fishing activity, the same pressures may not be present to exacerbate disease

development. Mortality has been found to correlate with a high intensity of infection in *O. edulis* (Robert et al. 2009). Class 1 infections were also low (8.3% & 9%, respectively) as it may be that once contracted *Bonamia ostreae* infection rapidly advances within the animal. The parasite was found in oysters of all weights but the highest prevalence of infection was found in the most common size group, 41 – 60 g, in both stocks, which is approaching market size. Prevalence of infection decreased in oysters larger than this, possibly due to mortality, fishing activity, these animals ability to suppress the development of heavy infections, tolerance of lower intensities of infection, or some ability to eradicate the infection. Several studies have observed that the impact of the disease was more evident in oysters with a higher weight. In the Netherlands, infection was most prevalent at ≥91 g (Engelsma et al. 2010) while in a previous Irish study, on the south coast, prevalence was also greatest in approximately 60 g oysters (Culloty & Mulcahy 1996).

B. ostreae was also found in oysters of all lengths but the highest prevalence of infection was found in size group 6.1 – 7.0 cm in both stocks. Infection progressively increased with growth at both sites until reaching 7.1 – 8.0 cm, after which prevalence of infection decreased. In the Netherlands, *B. ostreae* infection was also found to peak in flat oysters measuring 6.1 – 7.5 cm in length (Engelsma et al. 2010). The reason for the gradual decrease in prevalence after 60 g or 8 cm may be due to fishing activity, animals with a greater susceptibility to the disease having succumbed to the infection by this stage or that the larger, and thus older, animals may be undergoing some adaption or tolerance to *B. ostreae.*

In Clew Bay, though densities are low and so not likely to be a major stressor, the parasite persists. Overall the density of oysters in Lough Foyle is higher than Clew Bay's yet both stocks show relatively low oyster densities, compared to 5+ per m^2

recommended to have a viable oyster bed (Haelters & Kerckhof 2009). Density may be one factor contributing to development of infection in Lough Foyle but this association was not consistent between beds, indicating that it may be one of several factors impacting on disease development and density thresholds for the development of infection. Engelsma et al. (2010) found that, in Lake Grevelingen, Holland, where oyster density is > 1 per m^2, *B. ostreae* prevalence was independent of oyster density while other studies have found that prevalence of *B. ostreae* and density appeared to be related (Grizel 1985, Robert et al. 1991, Hudson & Hill 1991). In other species, such as *Ostrea chilensis,* high densities were shown to increase prevalence of the parasite *Bonamia exitiosa,* as crowding allowed for easier transmission (Hine et al. 2002). Doonan et al. (1999) found that the critical threshold density of oysters to trigger an epizootic was estimated to be ~1.26 oysters per m^2.

The Lough Foyle *O. edulis* fishery still remains operational, with the Southside bed (the location of Site A, B and C) being one of the main areas of activity. Indirectly the continual activity on this bed may be having an effect on prevalence of *B. ostreae* due to the continued disturbance and stress caused to the bed by fishing activity. Dredging of infected *O. edulis* beds can result in a much higher level of infection than on undredged beds (van Banning 1991). Similar observations have been made in *O. chilensis* with stress from vigorous stirring lowering the oysters' resistance to *B. exitiosa* (Hine al. 2002).

According to Wilson & Simons (1985), *O. edulis* reproduction begins when water temperatures are approximately 12 to 14°C on the west coast of Ireland. These temperatures were reached in Clew Bay and Lough Foyle in April 2011. Oysters at this time of the year are generally directing their energy towards gametogenesis, as spawning occurs during the summer months, thus they may have limited defences to

combat infection. Prevalence of infection was expected to be greatest around the period of initial spawning, usually June to July in Ireland, due to the reproductive stress placed on the animal; however Cáceres-Martínez et al. (1995) found no correlation between *B. ostreae* prevalence and gonadal development. da Silva et al. (2009) suggested that intense infections have a greater probability of occurring in females and even more so in ripe and recently spawned females. This may suggest that environmental aspects such as the increase in temperature have a direct influence on prevalence rather than the indirect effects of spawning stress. Other oyster species infected by *Bonamia* sp., such as *Crassostrea ariakensis,* have been found to be strongly influenced by temperature, with prevalence increasing as temperature increases (Carnegie et al. 2008).

In addition to spawning, by April the animals would possibly still be weakened from the previous winter stressors such as harsh environmental conditions or lack of food (Culloty & Mulcahy 1996). Engelsma et al. (2010) observed similar results in the Netherlands with the mean prevalence of infected oysters over the study period being significantly higher in spring than in autumn. Culloty & Mulcahy (1996) initially found prevalence to peak in autumn/winter in their study, thought to be related to initial post-spawning stress. The substantial reduction in prevalence in Lough Foyle, in July 2011, may be due to many animals, which became infected in the spring, succumbing to infection and thus decreasing prevalence overall.

The salinity in both stocks was similar, however the beds with the lowest mean prevalence of infection in both Clew Bay and Lough Foyle, Newport River (1.7%) and Flat ground (0.4%), were located beside large freshwater inputs. Previous salinity data gathered from Newport River, showed a range from 9.5 to 29.6 psu while in other beds such as Rosbarnagh the range was from 27.6 to 32.9 psu (M.

Hannan, *pers comm*.). Similarly a positive relationship between *Bonamia ostreae* prevalence and higher salinity has been observed (Engelsma et al. 2010) and Arzul et al. (2009) showed that, experimentally, higher salinity (≥35 g l^{-1}) supports better *B. ostreae* survival in *O. edulis*. Similar results have been found in other haplosporidians such as the parasite *Bonamia* sp. in *C. ariakensis,* with decreased prevalence being observed, at lower salinities, under a constant temperature in experimental conditions (Audemard et al. 2008). Extreme abiotic factors like salinity were shown to have an effect on *B. exitiosa* infected *O. chilensis,* increasing the spread of *B. exitiosa,* as high salinity may suppress the animal's immune defences (Hine et al. 2002).

In conclusion, the results appear to indicate that Clew Bay oysters, after 23 years of exposure to *B. ostreae*, still harbour infection, although heavily infected oysters are rarely observed. No form of management, in terms of breeding, occurs in the area. All the beds are self propagating thus the development of a naturally tolerant population may take many years, due to oysters which display some resistance continuously cross fertilising with susceptible oysters, or it may simply not be possible due to physiological constraints. However, results do show that a population of oysters can maintain itself for many years, albeit at lower densities, in the presence of *B. ostreae.* The susceptibility of *O. edulis* to infection with *B. ostreae* in both stocks seems to be related to a combination of factors including the size of the oyster, water temperature, density and salinity.

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Part B

Assessment of the impact of a pathogen, *Bonamia ostreae*, on differing North Sea *Ostrea edulis* stocks in Lake Grevelingen, Holland.

Abstract

Bonamia ostreae is a leading factor in the decline of *Ostrea edulis* populations throughout Europe. The parasite has spread throughout native Dutch stocks since its introduction to Holland in the 1980s while natural stocks in Denmark remain naïve to the disease. Hatchery bred spat from three stocks found in the North sea region, Lake Grevelingen and the Oosterschelde, Holland and Limfjord, Denmark, were placed in cages on a *B. ostreae* infected bed in Lake Grevelingen and screened bi annually for growth, mortality and prevalence of infection. The Lake Grevelingen stock had the highest overall prevalence of infection, yet it also had the greatest growth and survival rate of all three stocks. The Oosterschelde stock had a lower prevalence of infection but growth and survival rate were also low. The Danish stock had the lowest survival rate of all three stocks. Mortality levels may, in part, be due to the holding conditions of the oysters, as they prefer on-bottom culturing. As oysters were screened only every six months, it is difficult to determine what impact *B. ostreae* had on oyster mortality rates. As prevalence of infection was low each time oysters were screened, it would appear that local environmental factors and conditions may influence oyster growth and performance with local stocks better adapted to these conditions.

Introduction

Bonamia ostreae has been a leading factor in the decline of European *Ostrea edulis* populations since its introduction to the continent in the 1970s (Balouet et al. 1983, McArdle et al. 1991, van Banning 1991, Lallias et al. 2008). The stocks of *O. edulis* present in the Netherlands are located in two estuarine areas, the Oosterschelde and Lake Grevelingen. The Oosterschelde became infected with the protozoan pathogen in 1980, as a result of importations of infected oysters from France (van Banning 1988). Due to bonamiosis, the oyster production of the Oosterschelde suffered increasing losses and eventually the fishery ceased, as it was no longer commercially viable. The oyster stock of Lake Grevelingen has been screened since 1980 and was found to be free of *B. ostreae* until 1988. In the summer of that year, the first presence of the pathogen was observed. A rapid spread, with increasing prevalences followed, both in wild stock and in commercial stocks. In 1989 a maximum prevalence of infection of 48% and mortality rates of up to 80% were observed (van Banning 1991).

As of today, culture of flat oysters in the Oosterschelde is negligible, whereas in Lake Grevelingen annual production of flat oysters ranges from 50 to 100 tons (Engelsma et al 2010). With current *O. edulis* populations at only 3% of that in the 1970s, Dutch populations are under threat of being replaced by the Pacific Oyster, *Crassostrea gigas* (Haelters & Kerckhof 2009). The Danish Veterinary and Food Administration states that in Denmark, natural stocks of *O. edulis* only exist in the Limfjord region, where there is significant production. Aquaculture production of flat oysters is now also underway since 2000. *B. ostreae* has not been detected in native Danish populations to date.

As of yet, no method exists to eradicate the parasite once it becomes endemic within

a population, thus the Office International des Epizooties (OIE 2012) suggests the breeding of a "resistant" strain of *O. edulis* to combat the effects of *B. ostreae*. Selective breeding has been used in some stocks, such as Rossmore, Co. Cork, in an attempt to increase resistance to *B. ostreae* (Culloty & Mulcahy 2001). A previous attempt has also been made by Culloty et al. (2004) to identify naturally resistant stocks from the native stocks present in Europe, including Lake Grevelingen, by comparing them to selectively bred stocks. The results of that study suggested that differences exist between populations of *O. edulis* in susceptibility to infection with *B. ostreae*. The results also suggested that a degree of resistance has developed in the selectively bred Rossmore oysters. There was no evidence of increased resistance with respect to prevalence and intensity of infection in the Lake Grevelingen oysters. The aim of this study was to compare *O. edulis* stocks from the North sea region of Europe, with different histories of exposure to *B. ostreae,* to identify a well performing spat stock, which could potentially be used to repopulate flat oyster beds within the region. The growth rate, survival rate and susceptibility to bonamiosis in each stock were monitored in order to identify the best performing stock.

Materials And Methods

Study area

The study was carried out in Lake Grevelingen, South West Netherlands - a former estuary and now an artificially formed salt-water lake, connected to the North Sea by sluices. The lake has a surface area of 108 km^2. Approximately 60 % of its area is shallower than 5 m, though the deepest point is 48 m (Verhagen & Nienhuis 1983, Oorthuysen & Iedema 1989). *Bonamia ostreae* has been present in Lake Grevelingen since 1988 (van Banning 1991).

Sampling and screening of oysters

Oyster bed

All fieldwork and sampling of oysters was carried out by the Dutch Oysterecover partners, IMARES. Samples of live adult *Ostrea edulis* were collected from an *O. edulis* cultured bed in Lake Grevelingen, Holland, in October 2010 and October 2011 to determine the average prevalence of infection within the bed. Sixty individuals were collected from the bed on both sampling dates (n=120).

Stocks of spat

Spat from three *O. edulis* stocks were produced in a hatchery in early 2011 and placed in cages located on the cultured bed in Lake Grevelingen in April/May 2011. The three stocks of spat consisted of:

Cage O – Spat from parent oysters that came from the Oosterschelde estuary, a potentially tolerant or more resistant stock, as *B. ostreae* has been present in this stock since 1980.

Cage G - Spat from parent oysters that came from Lake Grevelingen, a long term infected stock, since 1988.

Cage D - Spat from the Limfjord, Denmark, a naïve stock, as no *B. ostreae* infection has been reported to date in native populations.

Each stock of spat was initially kept in a singular circular plastic cage, 73 cm x 20 cm. The cages were filled to one quarter of their volume with spat, 1100 spat in Cage G and 1200 spat each in Cages O and D. As the spat grew over the course of the study period, the oysters were moved into additional cages. Cages were also changed at frequent intervals to reduce the impact of fouling. From October 2011 to August 2013, samples of these spat stocks were collected from cages on five different dates (October 2011, April 2012, October 2012, April 2013 and August 2013). Survival rates were also monitored on each of these occasions.

A minimum of 18 to a maximum of 30 individuals were collected from each stock on each sampling occasion. The sampling size depended on the availability of oysters with a desired sample size being 30 individuals. A total of 420 spat were sampled from all of the cages (Table 1). Oysters were processed immediately upon arrival at the laboratory in Cork, with the whole wet weight (g) and length (cm) of each oyster recorded before oysters were opened.

Table 1. Number of oysters screened on each sampling date over the study period

Date (from start of trial)	Cage O	Cage G	Cage D	Total
Oct-11 (+ 5mths)	30	30	28	**88**
Apr-2012 (+11mths)	30	30	30	**90**
Oct-2012 (+17mths)	25	30	28	**83**
Apr-2013 (+23 mths)	30	30	27	**87**
Aug-2013 (+27 mths)	24	30	18	**72**
Total	**139**	**150**	**131**	**420**

Cytological processing and examination

Diagnosis of *Bonamia ostreae* infection was carried out on all oysters, using ventricular heart imprints, which were air dried before being fixed in methanol for two minutes, stained with Hemacolour 2 and 3 (Merck) and washed prior to mounting with DPX (Culloty et al. 1999). The intensity of *B. ostreae* infection was determined through classification of heart imprints from class 0, uninfected, to class 4, heavily infected (Bachére et al. 1982).

DNA isolation and polymerase chain reaction (PCR) analysis

A small sample of gill was collected from each oyster and stored at –20°C for PCR analysis. DNA was extracted from the tissue using the Chelex-100 method (Walsh et al. 1992, Lynch et al. 2008). The extractants were stored at –20°C. PCR analysis was performed with primers Bo Boas, which amplify 300 bp from the SSU rRNA gene, using the method of Cochennec et al. (2000). All PCR assays included positive controls (*B. ostreae* DNA) and a negative control (double distilled water). The PCR

products were run on 2% agarose gel in TE buffer gel (110V and for 40 minutes) stained with ethidium bromide (10mg/L).

Statistical analysis

The data were analysed with the software Microsoft Excel (Mac 2011 Version 14.2.3) and StatPlus:mac (AnalystSoft). One-way ANOVA tests were used to determine if there was a significant difference between the growth rate and survival rate of each of the three spat samples used in the trial.

Results

Screening of adult oysters from Lake Grevelingen

Heart imprints and PCR analysis performed on the October 2010 sample showed that the oysters from this cultured bed had a prevalence of infection of 16.7%, 13.3% confirmed by heart imprints with an additional 3.3% confirmed by PCR analysis (Fig. 1). Of the infected oysters, 50% (4/8) had class 2 infections, followed by class 3 (37.5% (3/8)) with one individual having a class 4 infection (Fig. 2). Heart imprint and PCR screening carried out on samples from the same bed in October 2011, show that the oysters had a prevalence of 15%, confirmed by heart imprint with no additional confirmations by PCR analysis (Fig.1). Of the infected oysters 44.4% (4/9) of the oysters had class 2 infections and 55.6% (5/9) had class 3 infections (Fig. 2).

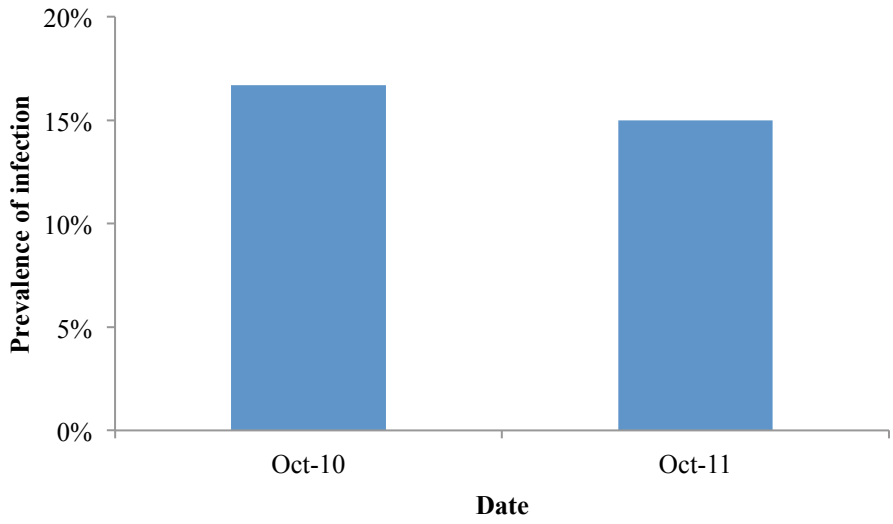


Fig.1. Prevalence of *Bonamia ostreae* (%) in Lake Grevelingen oysters, screened in October 2010 and October 2011.

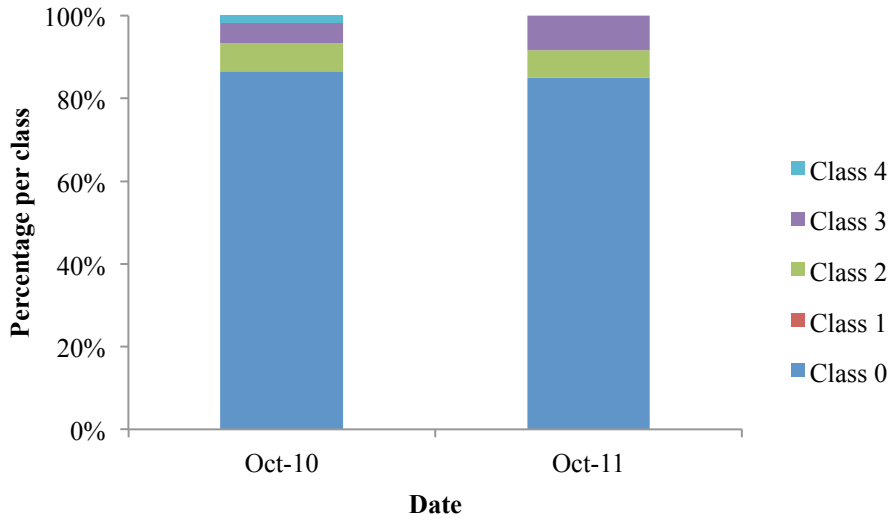


Fig. 2. Intensity of *Bonamia ostreae* infections observed in Lake Grevelingen oysters, screened in October 2010 and October 2011.

Screening of spat from cage trials

Weights and lengths of oysters

When the average weight of each spat stock over the sampling period was calculated Cage G, the Lake Grevelingen spat, had the greatest growth over the sampling period, having grown an average of 49.2 g. Cage D grew an average of 36.2 g while Cage O grew an average of 32.6 g, though the stock ceased to grow after October 2012 (Fig.3). Statistically differences between the three cages, in terms of weight growth, were insignificant ($F=0.55$, $p=0.59$).

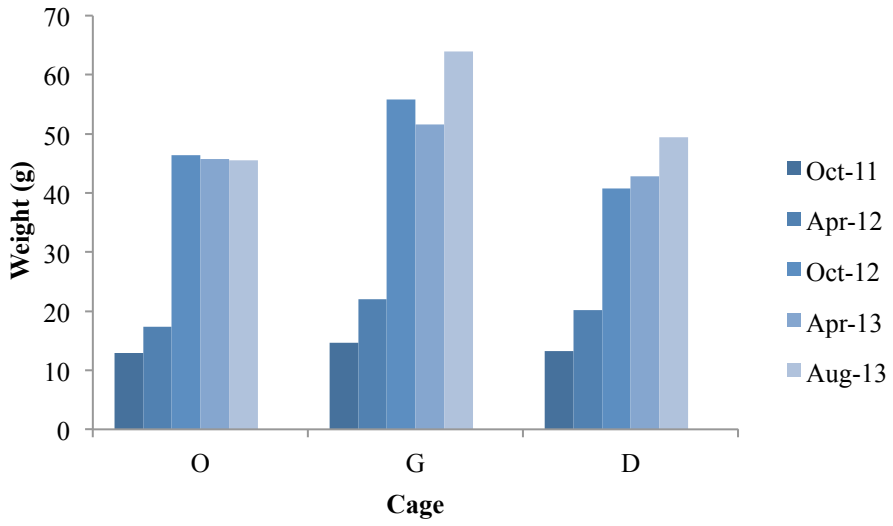


Fig.3. Average weight (g) of spat in cages per sampling date, with Cage O equating to Oosterschelde spat, Cage G to Lake Grevelingen spat and Cage D to Danish spat.

Similarly, when the average length of each spat stock over the sampling period was calculated, Cage G had the greatest growth over the sampling period, an average growth of 2.6 cm. Both Cage O and D grew an average of 1.8 cm (Fig. 4). Statistically differences between the three cages, in terms of length growth, were insignificant (F=1.93, p=0.19).

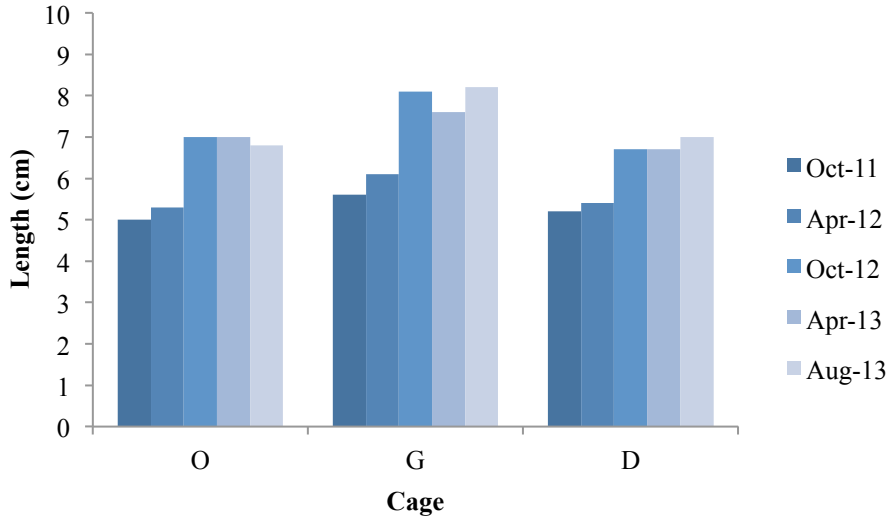


Fig.4. Average length (cm) of spat in cages per sampling date., with Cage O equating to Oosterschelde spat, Cage G to Lake Grevelingen spat and Cage D to Danish spat.

Spat survival

The number of surviving spat per stock was monitored over the course of the study. Again, animals in Cage G performed the best out of the three stocks with 15.6% (173/1100) spat surviving until the end of the trial while only 3.5% (42/1200) and 4% (48/1200) survived in Cage D and O respectively (Fig. 5). Statistically differences between the three cages, in terms of survival, were insignificant ($F=3.22$, $p=0.06$).

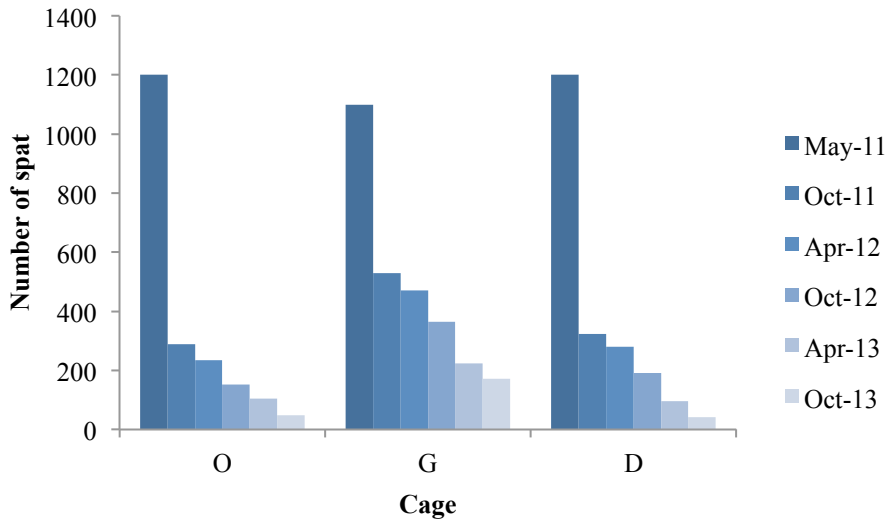


Fig. 5. Total number of live spat, from each, stock observed in cages over the study, with Cage O equating to Oosterschelde spat, Cage G to Lake Grevelingen spat and Cage D to Danish spat.

Prevalence and intensity of infection

When spat were initially screened in October 2011, 5 months post relaying, Cage O showed no infection (Fig. 6). Heart imprints were negative but PCR analysis performed on the spat from April 2012 showed that Cage O had a prevalence of infection of 3.3% (Fig. 6). The positive infections in Cage O were confirmed by PCR analysis thus intensity of infection could not be concluded (Fig. 7). Heart imprints and PCR analysis performed on the spat in October 2012 showed that 12% of Cage O oysters were infected (Fig. 6). Of these, 67% (2/3) had a class 2 infection while 33% (1/3) had a class 1 infection (Fig. 7). Heart imprints and PCR analysis performed on spat from April and August 2013 showed no apparent infection in Cage O (Fig. 6). Over the course of the study Cage O oysters had an overall prevalence at 3.1%.

When spat were initially screened in October 2011, Cage G showed no infection (Fig. 6). Heart imprints and PCR analysis performed on the spat from April 2012 show that 10% of Cage G samples were infected (Fig. 6). The single infected oyster confirmed by heart imprint, in Cage G, had a class 3 infection (Fig. 7). The additional two positive infections in Cage G were confirmed by PCR analysis thus intensity of infection could not be concluded (Fig. 7). All screening in October 2012 was negative for infection. Heart imprints and PCR analysis performed on spat from April 2013 showed Cage G had a prevalence of infection of 13% (Fig. 6). 50% of infected oysters had class 2 infections (2/4) while 25% (1/4) had a class 1 and another 25% had class 3 infections (Fig. 7). Heart imprints and PCR analysis performed on spat from August 2013 showed that Cage G had a prevalence of infection of 3.3% (Fig. 6). The single infected oyster confirmed by heart imprint, in Cage G, had a class 2 infection (Fig. 7). Overall, prevalence of infection throughout

the study was highest in Cage G stock at 5.3%.

When spat were initially screened in October 2011, Cage D oysters showed no infection (Fig. 6). Heart imprints were negative and PCR analysis performed on the spat from April 2012 show that Cage D had a prevalence of infection of 3.3%, rising to 7.1% in October 2012, as confirmed by PCR analysis (Fig. 6). Heart imprints and PCR analysis performed on spat from April 2013 showed that Cage D had a prevalence of infection of 7.4% (Fig. 6). The two infected oyster confirmed by heart imprint, in Cage D, had class 2 infections (Fig. 7). Heart imprints and PCR analysis performed on spat from August 2013 showed Cage D had a prevalence of infection of 5.6% (Fig. 6). Overall prevalence of infection, during the study, was 4.7% in Cage D oysters.

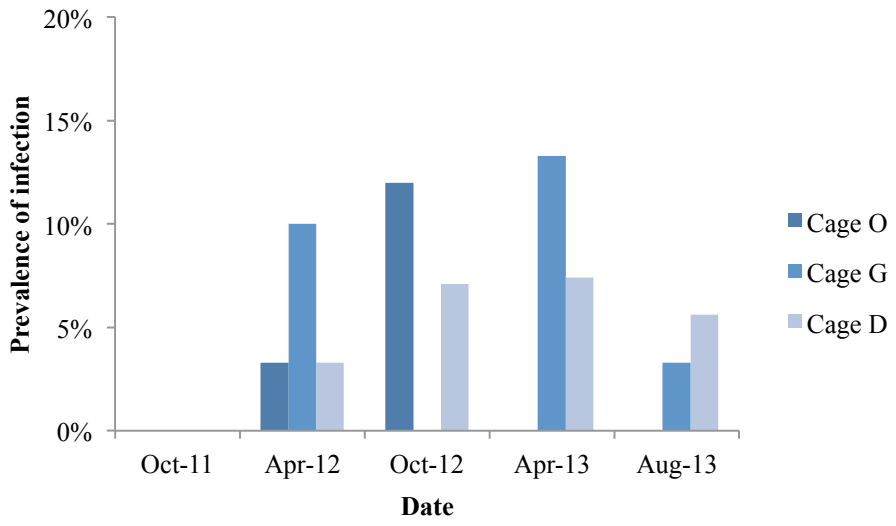


Fig. 6. Prevalence of *Bonamia ostreae* (%) in screened oysters from cages within Lake Grevelingen over the study period, with Cage O equating to Oosterschelde spat, Cage G to Lake Grevelingen spat and Cage D to Danish spat.

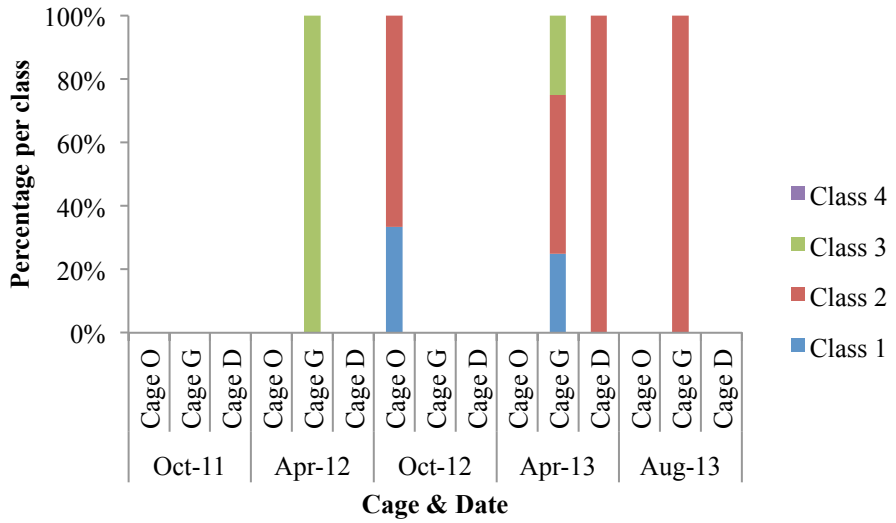


Fig. 7. Intensity of infection observed in different stocks held in cages in Lake Grevelingen over the study period, with Cage O equating to Oosterschelde spat, Cage G to Lake Grevelingen spat and Cage D to Danish spat. Cages with no information on intensity of infection had no infection determined by heart imprint.

Discussion

The prevalence of *Bonamia ostreae* infection in the adult oysters screened from the Lake Grevelingen flat oyster bed appears to be relatively stable, with approximately the same infection observed in October 2010 and in October 2011. In a previous study, Culloty et al. (2004) found prevalence of infection in Lake Grevelingen varied between 0% and 70%.

When cages placed on the same bed were screened for *B. ostreae* infection, it was noted that prevalence of infection was never detected above 15% in any of the stocks. The Lake Grevelingen (Cage G) stock had the greatest overall prevalence of infection in the screened oysters of all three stocks, indicating that, although native to the area, this stock was the most susceptible to the disease. Yet when mortalities were monitored for each stock, survival rates were much higher in the Lake Grevelingen stock compared with those from the Oosterschelde (Cage O) and Denmark (Cage D). This may suggest that these two stocks had succumbed to *B. ostreae* infection or other factors may be contributing to their low survival rate. It has previously been noted that Lake Grevelingen stock did not do well in terms of prevalence and intensity of infection, but performed well in terms of overall survival (Engelsma et al. 2010).

The Oosterschelde and Danish stocks had very low overall prevalences of infection, yet less than 4% of the spat survived in each stock compared to Lake Grevelingen, with 15.6% of the spat surviving, even though it had the highest overall prevalence of infection of the three stocks. Again, it may be that the majority of spat in Cage O and D succumbed to infection upon exposure to the parasite, or that *B. ostreae* is not the only factor impacting on the survival of the stocks. It is possible that local factors and conditions may impact on the stocks. Lake Grevelingen, as the native stock, has

probably adapted to these factors and thus performed the best of all three stocks. These factors may be very localized. Though located beside Lake Grevelingen, the spat stock from the Oosterschelde, which is genetically the same population as Lake Grevelingen, should have similar acclimation but it had a comparable survival rate to the Danish stock. As was expected, the number of spat in each stock cage declined over time. Mortality in oyster stocks can be high, mortality rates in the cultivation of oysters have noted to be as high as 80% (Cigarría & Elston 1997). Intensive oyster cultivation, such as in cages, may be more sensitive to disease and its effects (Paynter et al. 1992)

B. ostreae was found in the Oosterschelde stock only in April 2012 and October 2012 while it was found from April 2012 onwards in the Danish stock, possibly as a result of continued exposure of naïve oysters to the disease and continued development of new infection. The lack of infection observed in all three stocks in October 2011 may be due to the fact that the disease was still in its latent period, which can last for several months (Tigé & Grizel 1984, Elston et al. 1987, Montes 1991, Culloty & Mulcahy 1996, Culloty et al. 2001), as the spat were only placed on the bed in April and May of 2011. Mortalities were observed in all stock cages in June 2011 but particularly in Cages O and D. This was believed to be due to anoxic conditions caused by cage fouling but as stated above, it is possible that the mortalities in the cages were due to *B. ostreae* infection.

Prevalence of infection ranged from 0% to 13% in Lake Grevelingen stock with the greatest prevalences of infection being noted in April 2013 and April 2012, followed by August 2013 and October 2012. These results may indicate that as a native stock, seasonality is playing a major role in Lake Grevelingen stocks infection. As previously noted in Chapter 3, prevalence of infection can be highest in the late

spring possibly due to the stresses of spawning or a bad winter. Engelsma et al. (2010) also found that prevalence of infection in natural Lake Grevelingen stocks was highest in spring. Decreases in prevalence were noted in the Lake Grevelingen stock following these April peaks in prevalence of infection, indicating that mortalities may be occurring due to infection, reducing the number of infected individuals in the stock. Perhaps if sampling continued until October 2013, prevalence of infection would again be zero.

When growth rates were analysed it was again noted that the Lake Grevelingen stock performed the best, growing the most in weight and length of all three stocks of spat. The spat from the Danish stock were the smallest at the end of the study and the Oosterschelde stock ceased to grow after October 2012, perhaps again demonstrating a very favourable adaptation by the Lake Grevelingen stock to the local conditions.

In conclusion, it appears that of the three stocks used, Lake Grevelingen performed the best both in terms of survival and growth. Though Lake Grevelingen had the highest prevalence of infection of all three stocks, this prevalence was relatively low and growth rates were good, thus this is an acceptable balance. Further long-term field trials with more frequent sampling would be needed to confirm the good performance of the Lake Grevelingen stock and under more favourable husbandry conditions. The study appears to identify the fact that, with any attempt to culture oysters in an area, the stock used should be derived from native stock present in that area.

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Chapter 4

Part A

Investigating mechanisms for *Bonamia ostreae* to maintain itself outside of its host *Ostrea edulis*

Abstract

Transmission of *Bonamia ostreae* can occur directly from flat oyster to flat oyster but secondary mechanisms of transfer, even accidental, has not been completely ruled out. The means by which the parasite is transmitted are not yet fully understood. It has not been determined definitively, that oyster-to-oyster transmission is the only mode of transfer available to the parasite. Suspicion persists, however, that the disease may be spread by more means than just direct transmission between oysters. In several previous studies, it was found that even after the removal of all *Ostrea edulis* and a fallowing period of several years, oysters reintroduced into an area developed bonamiosis, indicating that a vector/reservoir of the disease may exist. To try to understand better how *B. ostreae* persists and maintains itself in the environment, screening of oyster beds, where *B. ostreae* is present, was carried out. Sediment, from an oyster bed infected by the parasite, was collected over a period of one year and screened using polymerase chain reaction (PCR) analyses to determine if life stages of *B. ostreae* could be detected, but results were all negative. A laboratory based transmission trial was also carried out, over a period of six months, whereby attempts were made to infect naïve *O. edulis* with *B. ostreae* using sediment from an area of known infection. All oysters used in the trial were screened for infection using heart imprints and PCR analysis. Sediment used in the trial was also screened using PCR analysis. No infection was detected in the oysters or sediment screened. The oyster beds were also screened to determine what macroinvertebrates were present and 14 different species were collected over a period of one year and screened using PCR analysis and *in-situ* hybridisation (ISH). Of the 14 species only one, *Mytilus edulis* demonstrated positive results - several samples of *M. edulis* were positive for the presence of *B. ostreae* using ISH and PCR analysis and *B. ostreae*-

like cells were observed in tissue sections. Additional individuals were collected in three other countries where *B. ostreae* is endemic but no infection was detected. The occurrence of *B. ostreae* within other bivalves may allow for the unintentional spread of the pathogen, especially with species such as *M. edulis*, which are frequently moved for farming purposes. The results of this study, plus previous studies, would indicate that *B. ostreae* may use a range of mechanisms and incidental carriers as a method of maintaining itself in the environment. When containing the disease, the results would suggest that all efforts should be made to reduce the possibility of transferring carriers from endemic areas.

Introduction

At present the complete life cycle of *Bonamia ostreae* remains unknown despite numerous studies on the issue (Montes et al. 1994, Culloty at al. 1999, Lynch et al. 2007). The means by which the parasite is transmitted or exists outside it's known host is not fully understood. It is known though that the disease can be directly transmitted from flat oyster to flat oyster (Elston et al. 1986, Hervio et al. 1995, Culloty et al. 1999), thereby indicating that an intermediate host is not required and *B. ostreae* can have a direct life cycle (Culloty & Mulcahy 2007, Arzul et al. 2009). The disease has also been shown to spread through cohabitation in the laboratory and inoculation with the parasite (Elston et al. 1986, Miahle et al. 1988, Hervio et al. 1995, Culloty et al. 1999). Montes et al. (1994) showed that *B. ostreae* enters a flat oyster through the gill epithelia, thus the parasite may enter an oyster during the filtration of seawater and/or respiration and continue to spread to other oysters via infected pseudo faeces (Bucke 1988, Montes et al. 1994) or through release into the water column from the tissue of oysters that have succumbed to infection (Culloty & Mulcahy 2007, Arzul et al. 2009). In the phylum Haplosporidia, which *B. ostreae* belongs to, a key characteristic of the group is spore formation. It is widely believed that an intermediate host is a necessary component of the life cycle in those species of Haplosporidia that form spores (Andrews 1984, Haskin & Andrews 1988, Powell et al. 1999, Burreson & Ford 2004).

Although a spore stage of *Bonamia ostreae* has yet to be discovered, suspicion still remains that the disease may be spread by more means than just direct transmission between oysters. van Banning (1988) found that even after the removal of all flat oysters and a fallowing period of several years, oysters reintroduced into Lake Grevelingen, Holland, developed bonamiosis, indicating that a vector/reservoir of the

disease may exist. The parasite has persisted for extended periods of time in various regions throughout Europe even when the oyster population has been significantly reduced (Lynch et al. *In press*). It is possible that sediment may act as a reservoir, or a macroinvertebrate species may act as an intermediate host or vector in the life cycle of *B. ostreae*. These suspicions were further fuelled with the discovery that the copepod *Paracartia grani* acts as a host of *Marteilia refringens*, another parasite of *O. edulis* (Audemard et al. 2002) and additional studies revealed six taxa of zooplankton to be hosts of the disease (Carrasco et al. 2007). *M. refringens* is also a protozoan parasite that effects *O. edulis* but belongs to the phylum Paramyxea (Berthe et al. 2000). It mainly infects the digestive tract with sporulation taking place in the digestive gland tubules and ducts (Grizel et al. 1974).

Most recently Lynch et al. (2007), using PCR analysis, found eight benthic macroinvertebrates and nineteen grouped zooplankton samples gave positive results for the presence of *B. ostreae* in an area where oysters have been infected with the disease since the early 1980s. Of the three macroinvertebrate species subsequently used in cohabitation trials, transmission was effected from brittlestars, *Ophiothrix fragilis*, to naïve *O. edulis.* Several previous studies looked at the ability of other macroinvertebrate species such as the Pacific oyster, *Crassostrea gigas*, mussels, *M. edulis* and *Mytilus galloprovincialis,* the edible cockle, *Cerastoderma edule,* and clams, *Ruditapes decussatus* and *Ruditapes philippinarum,* to act as carriers of *B. ostreae* but failed to detect it, though these studies only used microscopy for detection (van Banning 1987, Figueras & Robledo 1994, Culloty et al. 1999, Lynch et al. 2010).

It is also possible that an infective spore stage of *B. ostreae* may exist. As previously mentioned a characteristic of the Haplosporidia group, which *B. ostreae* belongs to,

is the presence of a spore stage (Burreson & Ford 2004). Thus far, of all the *Bonamia* species, only *Bonamia perspora*, which infects *Ostreola equestris*, has been found to possess a spore stage. It develops an ornamented spore with an orifice covered by a hinged operculum, in addition to sporocysts and sporonts, a typical characteristic of the haplosporidian genus *Haplosporidium* (Carnegie et al. 2006). Perhaps under more specific conditions other species of *Bonamia* may have the capability to sporulate.

The aim of this study was to further investigate mechanisms by which *B. ostreae* may be maintaining itself on oyster beds. The objective was to screen samples of sediment and macroinvertebrate species from an area of known *B. ostreae* infection using several diagnostic methods. This study based at Lough Foyle, an area where *B. ostreae* was initially diagnosed in oysters in 2006, follows on from a study by Lynch et al. (2007) in Cork harbour, the area in Ireland where *B. ostreae* was first confirmed. One objective was to determine if similar results would be found in Lough Foyle, as had been found in Cork harbour for the positive results in the macroinvertebrates. In a series of laboratory trials, naïve oyster were exposed over several months to sediment from an area of known *B. ostreae* infection to determine if the sediment might act as a source of infection. In addition, further samples were obtained from *B. ostreae* endemic areas in three other countries.

Materials And Methods

Screening of sediment from Lough Foyle, a *Bonamia ostreae* endemic area

The sediment samples were collected from Lough Foyle, a bay on the north coast of Ireland, a *B. ostreae* endemic area. Approximately 800 g of sediment was collected, by bottom dredging in a *B. ostreae* infected oyster bed, in Lough Foyle on five occasions – October 2010, January 2011, April 2011, July 2011 and October 2011. 200 g of the sediment from each month was initially passed through 25 μm retention filter paper using UV filtered seawater. The filtrate was subsequently passed through a series of filter papers - 15-18 μm retention filter paper, 7-12 μm paper, 2.5 μm paper and finally ≤ 2 μm paper. Each filter paper and a 50 ml sub sample of the final filtrate was collected and frozen. DNA extraction was performed on triplicates of each filter paper (n=75). DNA was extracted and PCR analyses were performed as outlined below.

Laboratory transmission trial

20 kilograms of sediment was collected using dredgers in Lough Foyle. All sediment was filtered, using large mesh-sized sieves (2 ml – 45 μm), to remove macroinvertebrates or plant matter before use. Three systems (A, B and C), each with 3 replicate tanks were set up, nine 100 L tanks in total. System A consisted of potential *B. ostreae* cells that had been released into the tank environment from infected Lough Foyle oysters to have a potential additional source of *B. ostreae* infection from the sediment. The 3 tanks in System A each contained 60 *Ostrea edulis* from infected Lough Foyle stock, and 3 kgs of sediment. The Lough Foyle oysters remained in the tanks for a period of 4 weeks before being removed and

screened for *Bonamia ostreae* infection. Naïve oysters from Loch Ryan, Scotland, 45 oysters per tank, were then added to the system. System B contained naïve oysters from Loch Ryan, 45 oysters per tank, and 3kgs of sediment. System C consisted of control tanks containing naïve oysters from Loch Ryan, 45 oysters per tank, with no sediment or substrate. A preliminary health screen was taken of the Loch Ryan oysters (n=60) using heart imprints and PCR prior to the trial commencing. Tanks were maintained at ambient temperature and oysters in each tank were fed once per week, 5 ml per system, with Instant Algae shellfish diet 1000®. A 20% water change was carried out every two weeks.

The trial ran for a period of six months, system A ran for 7 months in total (one month to hold the infected Lough Foyle oysters and 6 months for the transmission trial). Oysters that became moribund or died over this period were removed and screened for *B. ostreae* infection using heart imprints and PCR analysis. Upon completion of the trial, 200 g of sediment was taken from each tank and filtered using the same method as used for the seasonal sediment samples. DNA extraction was performed on triplicates of each filter paper (n=90, 6 sediment samples, five filters per sample and triplicate analysis of each filter). DNA extraction was also performed on triplicates of each filter paper using the Puregene tissue kit (Gentra Systems Inc.) extraction method (n=90). PCR analyses were carried out on all extracted DNA (n=180) to screen for the presence of *B. ostreae.*

Ventricular heart imprints

Each oyster was opened, the complete heart removed, placed onto tissue paper and the ventricular heart imprinted onto a slide. The imprints were air dried before being fixed in methanol for two minutes and stained with Hemacolour 2 and 3 (Merck)

(Culloty et al. 1999). The imprints were examined under light microscopy at 400x and 1000x magnifications. The intensity of *Bonamia ostreae* infection was determined using the scale devised by Bachère et al. (1982) and Culloty et al. (2004).

Screening of macroinvertebrates from *B. ostreae* endemic areas

Macroinvertebrates were collected with the sediment samples, in Lough Foyle on five occasions – October 2010 (n=76), January 2011 (n=48), April 2011 (n=37), July 2011 (n=31) and October 2011 (n=38). The samples were taken from oyster dredges carried out by commercial fishermen, who removed all of the macroinvertebrates present. Two hundred and six animals in total, consisting of 14 species, were collected over a 12 month period. Samples were catalogued and preserved in 70% ethanol. Each individual was prepared for PCR and *in-situ* hybridisation (ISH). Isolated tissue from each animal, either gill or gonadal tissue, was used for PCR. For ISH, small-sized species were divided into two parts while gill or gonadal tissue of larger species was isolated for screening. An additional sample of *Mytilus* spp. (n=100) was obtained from Lough Foyle in June 2013 and screened using the Chelex-100 and PCR methods outlined below.

Two sites (138 animals, 6 species) were screened in Brest and Quiberon, France in July 2012 and screened using PCR analysis. *Mytilus* spp. were also obtained from Brest and Quiberon, France (n=78), Lake Grevelingen, the Netherlands (n=60) and Ria de Arousa, Spain (n=60), four regions where *Bonamia ostreae* has been endemic since the 1980s. DNA analyses was carried out on all the additional mussel samples, using the Chelex-100 and PCR methods outlined below, to check for the presence of *B. ostreae*.

DNA extraction methods for laboratory and field screening

DNA was extracted from tissue or filter paper using the Chelex-100 method (Walsh et al. 1991, Lynch et al. 2008). The extractions were stored at –20°C. DNA was extracted from filter paper using the Puregene tissue kit (Gentra Systems Inc.) in accordance with the manufacturer's instructions. The extractions were stored at –20°C. DNA was extracted from paraffin-embedded mussel tissue found positive for *Bonamia ostreae* with *in-situ* hybridisation, using the QIAamp DNA FFPE Tissue Kit (Qiagen) in accordance with the manufacturer's instructions. The extractions were stored at –20°C.

PCR analysis for *Bonamia ostreae* infection

PCR analyses were performed with primers Bo Boas, which amplify a 300 bp from the SSU rRNA gene, using the methods of Cochennec et al. (2000). All PCR assays included positive controls (*Bonamia ostreae* DNA) and a negative control (double distilled water). The PCR products were run on 2% agarose gel in TE buffer gel stained with ethidium bromide. All results were categorised as either positive or negative.

PCR analysis for *Mytilus* spp. identification

PCR analyses were performed on all positive mussel samples with primers Me 15 and 16, which amplify a species-specific product of approximately 180 bp for *Mytilus edulis* and 126 bp for *Mytilus galloprovincialis*. Both products amplified in an individual indicate a hybrid of the two species (Inoue et al. 1995). The PCR products were run on 2% agarose gel in TE buffer gel stained with ethidium bromide.

In-situ hybridisation

An oblique transverse section was taken from each invertebrate samples in Lough Foyle, during the October 2010 – October 2011 sampling period, and fixed in Davidson's seawater fixative for 48 hours (Shaw & Battle 1957**,** Howard & Smith 1983). These tissue samples were then embedded in paraffin and cut into 8 μm sections. The sections were placed on silane-prep slides (Sigma AldrichTM). To create the probe, the primers Bo Boas, which target the SSU rDNA gene, were chosen (Cochennec et al. 2000). The probe was labeled with digoxigenin, incorporated by PCR. All solutions and buffers used were made up according to the protocol described by Sambrook and Russell (2001) and Lynch et al. (2008). The sections were examined under light microscopy at 400x magnifications. Results were categorised as either positive or negative.

Processing and examination of histological sections

For mussels found positive for *B. ostreae* by ISH (n=5), further 5 μm sections were cut and stained with haematoxylin and eosin. The sections were examined under light microscopy at 400x and 1000x magnifications. Results were categorised as either positive or negative for the presence of *Bonamia ostreae*.

Testing for the occurrence of inhibition during PCR analysis

To assess the possibility that inhibition may occur during the PCR analyses on the filtered sediment samples, triplicate samples of DNA (n=9) extracted from a sediment sample (1 μl) were spiked with *B. ostreae* positive *O. edulis* DNA (1 μl) from separate Class 1, 2 and 3 infected oysters and rescreened using PCR analyses.

Results

Screening of sediment from a *Bonamia ostreae* endemic area

A total of 75 samples and 1 kg of sediment, from the 5 months sampled from October 2010 – October 2011, were screened for the presence of *Bonamia ostreae* using the Chelex-100 extraction method and PCR analysis with Bo Boas primers. All samples were negative for *B. ostreae* DNA.

Laboratory transmission trial

Screening of the Lough Foyle oysters (n=156/180) placed in System A, using heart imprints and PCR analysis, showed an overall prevalence of infection of 10.3% upon completion of the 4 weeks, with heart imprints. All negative samples were further screened with PCR analysis but all remained negative. Prior to the trial commencing, an initial health screen of the naïve Loch Ryan oysters (n=60), using heart imprints and PCR, showed no *Bonamia ostreae* infection. Heart imprints carried out on the moribund Loch Ryan oyster, from all three systems (n=315/405), 79% (106/135) from system A, 76% (103/135) from system B and 79% (106/135) from system C were screened (Fig.1), over the course of the trial showed no evidence of infection. Certain oyster samples were unsuitable for screening due to the condition of the tissue. PCR analyses were also carried out on gill tissue from all the available moribund oysters (n=315). These were also negative for *B. ostreae* DNA. The majority of oysters were screened in July and August 2012, when ambient temperatures were at their highest (21.7°C), approximately 16 weeks after the trial commenced.

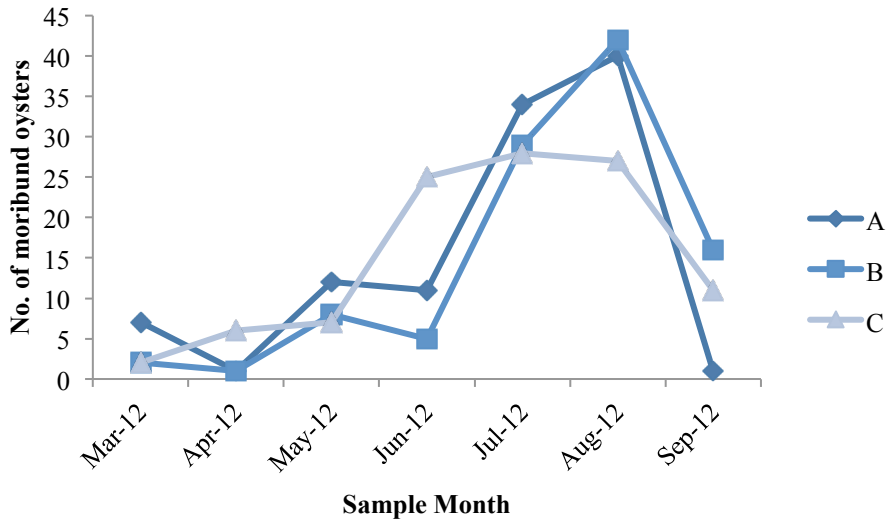


Fig.1. Number of moribund oysters removed from each system and screened per sampling month.

All screening of sediment, used in the transmission trials, from system A (n=45) and system B (n=45), were negative for *B. ostreae* DNA using the chelex-100 extraction method and PCR analysis. Samples were further screened (n=90) following extraction using a Gentra Puregene extraction method but all samples were still negative for *B. ostreae* DNA. All results were positive when sediment samples were spiked with classes 1 to 3 of *B. ostreae* (n=9), indicating that no inhibition occurred during PCR analyses, with no obvious difference in the appearance of bands between classes

Screening of macroinvertebrates, using PCR and ISH, from *B. ostreae* endemic areas

Of the 329 animals from 14 species (Table 1), from Lough Foyle, screened using PCR analysis and ISH, all were negative for *B. ostreae* infection except for five *Mytilus edulis*, out of a total of 142 mussel samples. All positive mussels were

sampled in Lough Foyle in October 2010. These mussels demonstrated *B. ostreae* like cells intracellularly (Fig. 5b) and were also found to stain positive for *Bonamia ostreae,* using ISH (Fig. 3b). Samples were compared to histological slides of uninfected *M. edulis* (Fig. 5a) infected *Ostrea edulis* (Fig. 4b) and uninfected *O. edulis* (Fig. 4a).

Using ISH *B. ostreae* positive reactivity was found distributed throughout the tissues. Samples were compared to ISH slides of uninfected *M. edulis* (Fig. 3a) infected (Fig. 2b) and uninfected *Ostrea edulis* (Fig. 2a). All of the positive Lough Foyle *Mytilus* samples were confirmed as *M. edulis.* The macroinvertebrates collected from France in July 2012 all screened negative for *B. ostreae* using PCR analysis (Table 2).

Table 1. Summary table of macroinvertebrate species and numbers of samples collected over the study period at Lough Foyle.

Species	Oct-10	Jan-11	Apr-11	Jul-11	Oct-11	Jun-13	Total
Mytilus spp.	28	10		3	1	100	142
Ascidiella aspersa	16	7		6	3		32
Palaemon serratus	10	7	9	3	6		35
Carcinus maenas	10	8	10	3	9		40
Ophiothrix fragilis	3				1		4
Asterias rubens	2	3	2	2	2		11
Galathea squamifera	2	9		6			17
Pagurus bernhardus	2	3	6	3	5		19
Maja squinado	3		8		8		19
Cerastoderma edule				1			1
Buccinum undatum			1	3			4
Psammechinus miliaris					1		1
Crossaster papposus			1		2		3
Aequipecten opercularis		1					1
Total	76	48	37	30	38	100	329

(a) (b)

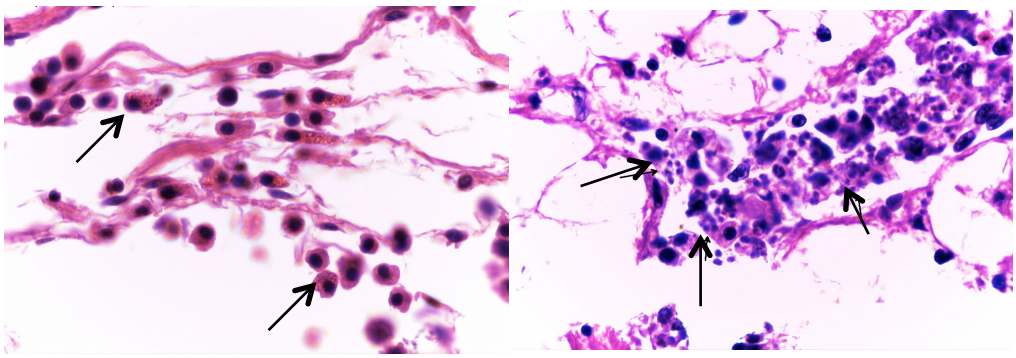
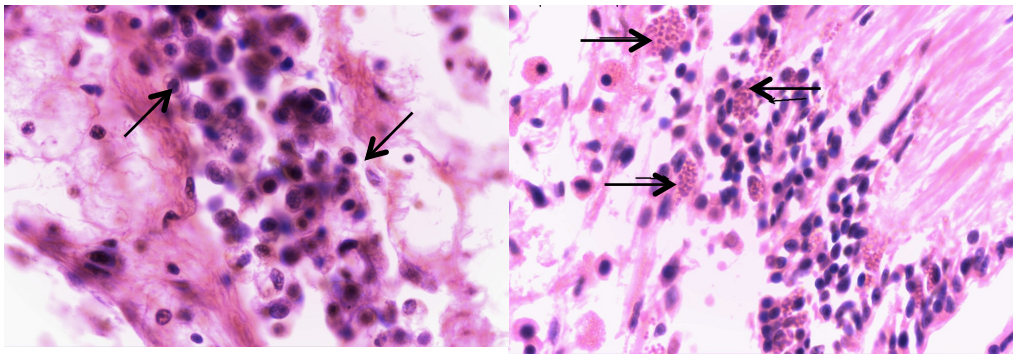


Fig. 2. Light microscopy of a histological section of (a) uninfected *Ostrea edulis* connective tissue and haemocytes (arrow) and (b) *Ostrea edulis* connective tissue infected by *Bonamia ostreae* cells (arrows).

(a) (b)

Fig. 3. Light microscopy of a histological section of (a) uninfected *Mytilus edulis*



tissue and haemocytes (arrows) and (b) *Mytilus edulis* infected by *Bonamia ostreae*-like cells (arrows).

(a) (b)

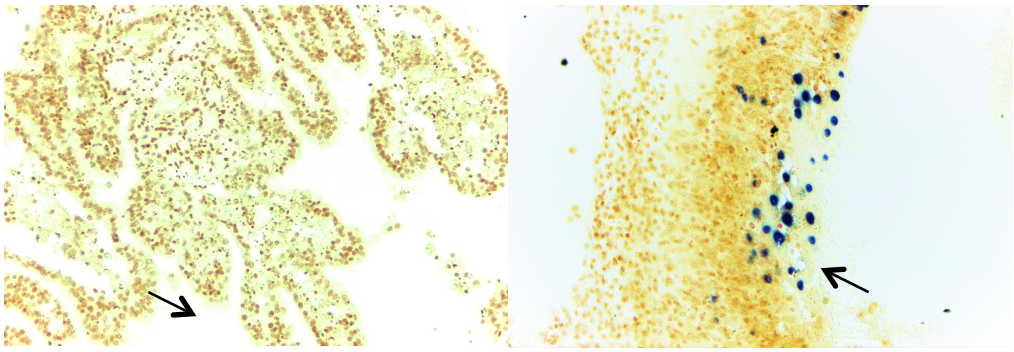


Fig. 4. Light microscopy of an *in-situ* hybridization section of (a) uninfected *Ostrea edulis* gill tissue and haemocytes (arrow) with no DIG labelled cells (b) *Ostrea edulis* with dark staining indicative of the presence of *Bonamia ostreae* cells in the connective tissue (arrow).

(a) (b)

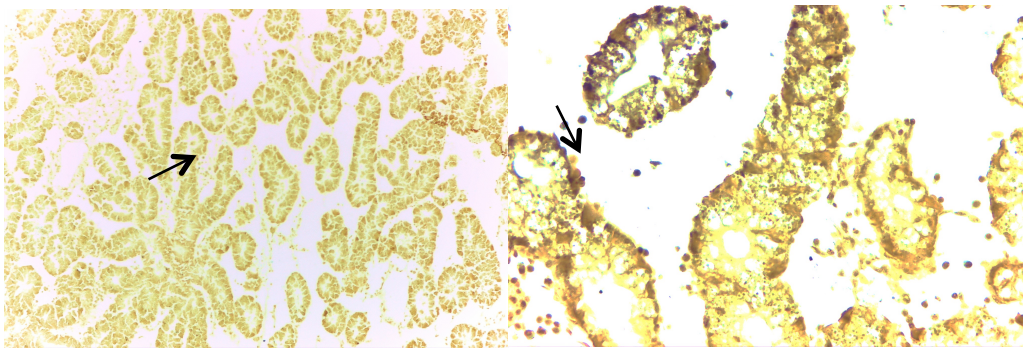


Fig. 5. Light microscopy of an *in-situ* hybridization section of (a) uninfected *Mytilus edulis* digestive tubule tissue (arrow) and haemocytes with no DIG labelled cells (b) *Mytilus edulis* with dark staining indicative of the presence of *Bonamia ostreae* cells in the digestive tubule tissue (arrow).

Table 2. Summary table of macroinvertebrate species, sample sizes and location of species collected in France, Spain and Holland.

Species	Brest	Quiberon	Spain	Holland	Total
Pecten maximus	6	6			12
Asterias rubens		1			1
Pagurus bernhardus	63	3			66
Liocarcinus holsatus	42	12			54
Atelecyclus rotundatus		5			5
Mytilus spp.	49	29	60	60	198
Total	160	56	60	60	336

Initial PCR analysis from chelex-100 extracted gill samples was negative. After five mussel samples showed positive for infection using ISH, DNA was re-extracted from the five relevant paraffin-blocked tissue using the QIAamp DNA FFPE tissue kit and PCR analysis was again carried out to confirm the presence of *B. ostreae*. All five samples had products amplified at the expected product size for *B. ostreae* (300bp). These products were sent for analysis but sequencing was unsuccessful, possibly due to the fragmentation of the DNA and the reduction of DNA quality during the histology process.

Of the additional samples of *M. edulis* obtained from Lough Foyle, DNA was extracted from the gonad, gill and mantle, using the Chelex-100 method but all results were negative (n=300). PCR analyses were carried out on the DNA extracted from the gill of the French, Spanish and Dutch *M. edulis* samples but results were negative for *B. ostreae* DNA.

Discussion

In this study, sediment and macroinvertebrate samples from a known area of *Bonamia ostreae* infection were screened for the presence of the parasite to determine their status as reservoirs or possible carriers of *B. ostreae*. There was no evidence of *B. ostreae* DNA in sediment from field screening or laboratory trials. In the field screening a small number of mussels were positive for *B. ostreae*-like cells, which also gave positive results when screened using molecular techniques. Questions still remain as to whether sediment can act as a reservoir and macroinvertebrates, other than oysters, as carriers (intentional or accidental) of the parasite (Culloty et al. 1999, Lynch et al. 2007). van Banning's (1988) study, which found that after the removal of all flat oysters and a fallowing period of several years, reintroduced oysters developed bonamiosis, suggested the possibility of the long-term persistence of *B. ostreae* outside its host. Also, *B. ostreae* has been shown over the last forty years to infect many other species of oysters including *Crassostrea angulata* (Carnegie & Cochennec-Laureau 2004), *Ostrea chilensis* (Grizel et al. 1983), *Ostrea puelchana* (Pascual et al. 1991), *Crassostrea ariakensis* (Cochennec et al. 1998), *Crassostrea gigas* (Lynch et al. 2010) and *Ostrea angasi* (Corbeil et al. 2009) so it is conceivable that the parasite can infect other invertebrates. A quarter of the species identified as Haplosporidia, the phylum to which *B. ostreae* belongs, are found infecting annelids (Siddall & Aguado 2006). A recently discovered haplosporidian of the polychaete *Syllis nippononica* was identified, by phylogenetic analysis, to belong to mollusk specific taxa and a haplosporidian-like infection was found in the edible crab, *Cancer pagurus* (Siddall & Aguado 2006, Thrupp et al. 2013). These studies add support the theory that *B. ostreae* can infect other invertebrates.

In this study no presence of *B. ostreae* was detected in the sediment or successfully transmitted from sediment to naïve oysters. This may be due to the fact that *B. ostreae* does not have a spore stage, it might be one of several species of Haplosporidia that may have abandoned production of spores (Carnegie et al. 2006), or it may not have a long living external life stage and thus cannot be transmitted through sediment (Arzul et al. 2009). It may also be difficult to extract DNA from spores only, thus if spores were present in the sediment they may have remained undetected, Rose et al. (2011) concluded that a heat step at the beginning of the process gave better results at breaking down spores than other approaches such as bead beating or chemical lysis. The failure of any oyster though to become infected indicates that no *B. ostreae* cells were present or stages in the sediment may only become infective under particular conditions. Oyster mortalities did occur during the sediment trial, this was most likely due to temperature increases in the systems as the tanks were maintained at ambient temperature. The rate of mortality increased over the summer months, as the surrounding temperature would have increased.

The novel idea of sediment as a reservoir for *B. ostreae* arose through the work on reservoirs of other parasites of bivalves, such as *Marteilia sydneyi,* of the phylum Paramyxea, which infects the Sydney rock oyster, *Saccostrea commercialis,* and whose spores can survive for up to 35 days outside a host (Roubal et al. 1989, Wesche et al. 1999). All *Bonamia* species have been placed in a phylum of spore-forming protists, Haplosporidia, though as of yet only *Bonamia perspora* has been shown to produce spores (Carnegie et al. 2000, Cochennec et al. 2000, Carnegie et al. 2006). A recent study by Carnegie et al. (2006) suggests that other species of *Bonamia,* including *B. ostreae,* may have the capability to sporulate under more specific conditions, though no spore stage has yet been observed in *B. ostreae*.

Studies on some spore producing haplosporidians have shown that they may be used as a means to transmit the parasite to new hosts, as seen in *Haplosporidium nelsoni*. *H. nelsoni*, also known as MSX, can cause large mortalities in the Eastern oyster, *Crassostrea virginica*. Similar to *B. ostreae,* the complete life cycle and means of transmission of the parasite remain unknown though the species is spore-forming (Barber et al. 1991, Stokes et al. 1997). To help elucidate the life cycle, a study by Stokes et al. (1997) collected sediment samples from Chesapeake Bay, an area with a high prevalence of *H. nelsoni*. These samples screened positive for the presence of the parasite using PCR analyses. It was suspected that *H. nelsoni* spores were widely distributed in the area and thus the cause of the positive results. Ford et al. (2008) found positive signals for the presence of *H. nelsoni* in the faeces and pseudo faeces of *C. virginica* and in the faeces of the ribbed mussel, *Geukensia demissa*. Transmission of the parasite has now been confirmed to be caused by the involvement of water-borne infectious particles and not directly by spores (Sunila et al. 2000).

In the field screening, of the 20 species collected during a similar study by Lynch et al. (2007) on the south coast of Ireland, only four of these species were found in the sampling of the Lough Foyle area, *Ascidiella aspersa, Carcinus maenas, Mytilus edulis* and *Ophiothrix fragilis.* The quantity of those species found were also much smaller than those in Lynch et al. (2007), for example, only four *O. fragilis* individuals were collected in Lough Foyle, all negative, compared to 54 in Rossmore of which 2 were positive. The range of macroinvertebrates available from Lough Foyle was limited to 14 species, possibly due to the active dredging of the area for *Ostrea edulis* in combination with the northerly location of the site and the mixture of poorly sorted sands, gravels and mudflats on the seabed, making the area

uninhabitable for certain species. Lynch et al. 2007's study found 8 macroinvertebrate species gave positive PCR results for *B. ostreae* DNA, these included a single *A. aspersa,* a single *C. maenas* and four *O. fragilis,* which all provided negative PCR and ISH results for the presence of *B. ostreae* when screened in this study. Prevalence of infection in oysters was similar at both sites during the year of sampling, 9% in Rossmore (Lynch et al. 2007) and 11% in Lough Foyle (Chapter 3 - Part A).

A total of 330 macroinvertebrates were screened from Ireland during this study, of which only five individuals tested positive for *B. ostreae* – five *M. edulis* samples from October 2010 were found to have *B. ostreae-like* cells within their tissues using ISH. Further screening using histology and PCR analyses confirmed the presence of intracellular microcells. While positive results for *B. ostreae* were found by ISH and PCR analysis, no sequencing of the products was possible. Though the OIE approve both PCR analysis and ISH as methods of confirmation for this parasite, further screening and analysis would be required to assess the extent to which mussels in Lough Foyle contain microcells. Prevalence of *B. ostreae* in Lough Foyle was 14.9% during that month of sampling (Chapter 3 – Part A), though there was a spike in *O. edulis* mortalities, up to 75%, in autumn 2010 due to *B. ostreae* infection (Loughs agency 2011).

As *M. edulis* are filter feeders (Hawkins et al. 1996) it is possible that as they filtered large amounts of seawater during these autumn months, they in turn ingested large quantities of *B. ostreae* cells. Similarly Lynch et al. (2007) concluded that as *Sabella pavonina*, *A. dissimilis, Actinia equine* and *Crassostrea gigas* are active suspension feeders, they may have ingested *B. ostreae* while feeding on suspended particulate matter and plankton, though in a further study on *C. gigas* (Lynch et al. 2010) the

parasite was also found intracellularly in a small number of animals. Further screening of *Mytilus spp.* samples from countries profoundly affected by *B. ostreae* over the past 30 years, France, Spain and the Netherlands, failed to detect any trace of *B. ostreae,* though in the areas where the samples were collected, prevalence of infection can be very high, in Ria de Arousa, Spain, up to 50%+ (A.Villalba, CIMA, *per comm.),* Lake Grevelingen, the Netherlands, up to 37% (P. Kamermans, IMARES, *per comm*.) Brest, France, up to 41% and Quiberon, France, 52% (A.Villalba, CIMA, *per comm*.). Perhaps a sudden, large deposit of *B. ostreae* cells in the water column is needed to permit uptake by mussels or that a small number of individuals are susceptible to the disease. Older studies deduced that *Mytilus* species did not carry the pathogen, yet these studies did not use any molecular techniques for screening, thus they could not accurately check for the presence of *B. ostreae (*Figueras & Robledo 1994, Culloty et al. 1999). Previous studies have shown that using a combination of more than one technique, including PCR, increases the probability of detecting *B. ostreae* (Lynch et al. 2008). A transmission trial, similar to Lynch et al. (2007), using *M. edulis* and several diagnostic techniques, would allow for a clearer representation of the ability of this microcell to replicate and be transmitted back to *O. edulis.*

The possibility that mussels may act as an incidental carrier of *B. ostreae* raises some important issues, considering that they are frequently farmed near flat oyster beds throughout Europe. As a result of farming, consignments of mussels are often moved within countries and between countries, from *B. ostreae* infected areas to uninfected areas. If mussels do indeed act as a carrier for the parasite these actions would allow for the further spread of the disease in Europe and/or may have been the cause of earlier unintentional transmissions of the disease. Further research is needed into the

issue to allow for proper management of bonamiosis. Great care and attention is required in all aspects of flat oyster farming to reduce the risk of contamination from infected beds via sediment, water and animals. Caution must be enacted to prevent the increased mortality in already infected populations and to prevent the diseases' spread.

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Part B

Investigating the significance of the role of larvae in the uptake and transfer of *Bonamia ostreae*

Submitted for publication as follows

Investigating the significance of the role of larvae in the uptake and transfer of *Bonamia ostreae*

A short communication

Grace Flannery, Sharon A. Lynch, Sarah C. Culloty

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In review

Abstract

Though most transmission of the protozoan parasite *Bonamia ostreae* occurs directly from oyster to oyster and the occurrence of vertical transmission from infected parent to brooding larvae has also been recently reported, the full range of mechanisms by which oysters become infected needs some study. In this study, the ability of oyster larvae, being brooded in the shell cavity of the parent oyster, to pick up infection from the surrounding environment was investigated. Samples of larvae were collected from brooding oysters over the course of three summers from three different areas around Ireland. Larvae were prepared and washed to ensure that no external *B. ostreae* was incidentally attached and the samples were screened for the presence of *B. ostreae* DNA using PCR analysis. Four samples of larvae from four oysters were found to be positive for *B. ostreae* DNA, though the corresponding parent oysters were negative for infection by PCR and by heart imprints. These results indicate that larvae may be able to acquire the pathogen from the water column during the process of filter feeding or elimination of pseudo-faeces by the brooding adult, even when the parents themselves are not infected. This has implications for hatchery production of larvae, larval transportation and mechanisms of transmission of *B. ostreae.*

Introduction

Bonamia ostreae has been the main parasite impacting on the European flat oyster, *Ostrea edulis,* since its discovery in the 1970s (Lynch et al. 2007). Though direct transmission of *B. ostreae* can occur from oyster to oyster, other vectors or reservoirs of infection, even incidental, may occur in different systems (Culloty et al. 1999, Lynch et al. 2007, Lynch et al. 2010). Early studies on this disease indicated it was a disease of older, larger oysters (Culloty & Mulcahy 1996) yet a more recent study discovered that young prespawning flat oysters are in fact susceptible to infection by *B. ostreae* (Lynch et al. 2005).

Arzul et al.'s (2011) study detected the presence of *B. ostreae* in larvae, from infected broodstock, through the use of PCR analysis and *in-situ* hybridisation, inferring that vertical transmission, from parent to offspring, could occur. Female *O. edulis* are larviparous. Fertilisation occurs in the mantle cavity and brooding eggs are incubated, for 8 to 10 days, to an advanced larval stage before being released (Lapegue et al. 2006). *O. edulis* seed production in hatcheries have reported sudden and unexplained larval and post-metamorphosis mortalities, usually considered due to bacterial contamination and flow-through techniques (Gonzalez Araya et al. 2012). The role of pathogens or disease in many of these cases has not been explained or investigated. In Ria de Viga, Spain, where *B. ostreae* is present, the survival rate in the cultivation of larvae from 2013 ranged from 0 to 8.1% (J. Fuentes, ECIMAT, *pers comm*.) while in Lough Foyle, Co. Donegal, it was noted that recruitment increased two-fold on a bed when *B. ostreae* prevalence dropped from 80 to 30% (C. McGonigle, Loughs Agency, *pers comm*.), indicating that larval mortalities could be in part due to *B. ostreae* infection.

It is now clear that all age groups of *O. edulis* can become infected with the parasite

yet the mode of transmission is not yet completely clear. It is possible that *B. ostreae* can be transmitted by plankton, benthic macroinvertebrates or by cells contained in the oyster pallial cavity (Lynch et al. 2007, 2010). Arzul et al. (2011) demonstrated that vertical transmission can occur but the ability of brooding larvae to become infected by another source, such as *B. ostreae* present in the surrounding environment, has not been established.

The objective of this study was to determine if infected brooding larvae are always associated with infected parents or if a secondary source of infection from the surrounding environment might allow for transmission to these larvae. The study assessed the extent to which infected larvae might contribute to the transmission of *B. ostreae* by looking at prevalences in brooding oysters over three summers.

Materials and methods

Over the summer period 2006, 2011 and 2012 sampling of adult *Ostrea edulis,* in three areas where *Bonamia ostreae* is endemic, in Ireland, took place (Table 1). The oysters were obtained from oyster growers at three locations and at times during the summer when the oyster growers considered there was opportunity to obtain brooding animals with larvae. In total, 150 oysters were screened in June 2006, 320 oysters in July 2011 and 180 oysters in July 2012 (Table 1). All oyster samples were opened and any *O. edulis* found to be brooding were identified. A sample of larvae was collected from each brooding oyster. Tissues (5 mm of gill tissue and ventricular heart tissue) were collected from the corresponding parents for heart imprints and standard PCR analysis. A total of 13 oysters, out of 150 (8.7%), with brooding larvae were collected from Rossmore, Co. Cork, during summer 2006. A total of 57 oysters, out of 320 (17.8%), with brooding larvae were collected from the three areas - Clew Bay, Co. Mayo, Lough Foyle, Co. Donegal and Rossmore, Co. Cork, during summer 2011. A total of 24 oysters, out of 180 (13.3%), with brooding larvae were collected from the same three areas during summer 2012 (Table 1).

Table 1. Number of oysters screened, number and percentage of brooding oysters and study sites over the study period during the summers of 2006, 2011 and 2012.

	Summer 2006	Summer 2011			Summer 2012		
	Rossmore	**Clew Bay**	**Lough Foyle**	**Rossmore**	**Clew Bay**	**Lough Foyle**	**Rossmore**
Sample size	150	60	60	200	60	60	60
Brooding oysters	13 (8.7%)	20 (33.3%)	16 (26.7%)	21 (10.5%)	10 (16.7%)	0 (0%)	14 (23.3%)

Screening of the larvae from all adults, infected or uninfected, was undertaken using standard PCR with primers Bo Boas, using the method of Cochennec et al. (2000). The larvae were washed by centrifuging them for five minutes, five times with double distilled water prior to screening, to ensure that any incidental *B. ostreae* cells attached to the larvae were removed. DNA was extracted using a Gentra® Puregene kit. DNA was quantified using a Thermofisher NanoDrop spectrophotometer. The parents were screened by heart imprint to diagnose clinical infection (Bachére et al. 1982, Culloty et al. 1999) and chelex-100 extracted gill DNA (Walsh et al. 1991, Lynch et al. 2008) was screened using standard PCR to investigate the presence of *B. ostreae* DNA (Cochennec et al. 2000).

Results and discussion

In 2006, all parent oysters from Rossmore (n=13) were uninfected. In 2011, all parent oysters from Clew Bay (n=20) and Lough Foyle (n=16) were also uninfected while 4.8% (n=1/21) of the Rossmore parent oysters were infected. Four samples of larvae, from four individual parents from Clew Bay, Co. Mayo, were found to be positive for *Bonamia ostreae* DNA, with products amplified at the expected size of 300bp. Low levels of 8.36, 8.47, 10.04 and 15.24 ng per µl of DNA were present in the four samples. Direct Sanger sequencing was performed and DNA BLASTn analysis showed sequence identity values of 99% with *B. ostreae*. The corresponding parents were not infected with *B. ostreae*, as confirmed by heart imprints and PCR. *B. ostreae* was detected in a single brooding Rossmore *Ostrea edulis* that same year, the corresponding larvae was negative for infection. In 2012, *B. ostreae* was detected in two parent Rossmore *O. edulis* but corresponding larvae were negative for infection. PCR analyses carried out on all other larval samples were negative for *B. ostreae* DNA (Table 2).

Table 2. Summary table of larvae and oyster samples screened for *Bonamia ostreae* infection in each location and year with those diagnosed positive in bold.

	Summer 2006	Summer 2011			Summer 2012		
	Rossmore	**Clew Bay**	**Lough Foyle**	**Rossmore**	**Clew Bay**	**Lough Foyle**	**Rossmore**
Parent oysters	13/150 (8.7%)	20/60 (33.3%)	16/60 (26.7%)	21/200 (10.5%)	10/60 (16.7%)	0/60 (0%)	14/60 (23.3%)
Infected parent oysters	0/13 (0%)	0/20 (0%)	0/16 (0%)	**1/21 (4.8%)**	0/10 (0%)	0/0 (0%)	**2/14 (14.3%)**
Infected larvae	0/13 (0%)	**4/20 (20%)**	0/16 (0%)	0/21 (0%)	0/10 (0%)	0/0 (0%)	0/14 (0%)

Vertical transmission may occur in *O. edulis,* yet in this study, none of the larvae found to be infected with *B. ostreae* had infected parents screened by heart imprints

and PCR analysis. Additionally, infected brooding oysters had uninfected larvae in their pallial cavities. Multiple washes were carried out prior to larval extraction to ensure all larvae were free of external DNA and so any detected *B. ostreae* was from inside the animal. These results indicate that larvae may be able to acquire the pathogen from the water column. One mechanism for this may be following exposure to *B. ostreae* in the pallial cavity, as a result of filter feeding by the brooding adult. Lynch et al. (2010) have shown that *B. ostreae* DNA can be detected in an oyster's pallial fluid. Evidence suggests that brooding larvae begin feeding while still in the parental cavity (Helm et al. 2006) though little is known of the size particles they can ingest. Arzul et al. (2011) found the parasite in the epithelia surrounding the visceral cavity of the infected larvae, suggesting that if they do, in fact, become infected with *B. ostreae* they do so through ingestion of the parasite. In the adult, the parasite may likely be actively ingested and has been found in pseudofaeces (S.C. Culloty, *pers comm.*). However, it appears that in adult oysters, at least one other focus of initial infection can be the gills where the parasite may enter actively through the epithelium or be taken up through this tissue (Bucke 1988, Montes et al. 1994). Parent oysters may need to be heavily infected for vertical transmission to occur.

An infection in *O. edulis* larvae may come from the infected pseudo-faeces of the parent oyster (Montes et al. 1994) or from *B. ostreae* cells in the water column, which are released from the tissue of oysters that succumb to infection (Arzul et al. 2009). Prevalence of infection was 12.3% in the Clew Bay population from which the larvae were sourced at the time of sampling (Chapter 3 – Part A) thus *B. ostreae* cells would be present in the environment. Prevalence of infection was on average 13% in the Bay of Quiberon where Arzul et al. (2011) sampled.

These findings may have implications for the farming of *O. edulis* in infected areas. If flat oysters are being bred in hatcheries in *B. ostreae* infected areas, such as France and Spain, these hatcheries may need to employ filtration systems for their incoming seawater supply to lower the exposure of larvae to *B. ostreae* from the external environment. *Haplosporidium nelsoni* and *Perkinsus marinus* have not yet been detected in the larvae of *Crassostrea virginica*, but as hatcheries are often in areas where both parasites are endemic, similar to *O. edulis*, it is recommended that all water is treated using a filter system and UV irradiation to lower any potential risk of infection (Ford et al. 2001). Caution should also be exercised when moving veligers as by this stage they may already be infected. The results imply that *O. edulis* are susceptible to *B. ostreae* infection prior to metamorphosis, thus *O. edulis* larvae should be considered as a possible source of *B. ostreae* infection in new areas, as larvae can travel on currents for as far as 12km (Wilson 1987). Further research is required on the potential movement of larvae. This study indicates that all life stages of *O. edulis* in *B. ostreae* infected areas must be managed with caution, to prevent increased mortality in hatchery populations and further spread of the disease in wild populations. Additional research into the exact cause of such large losses at larval stages, as mentioned by Gonzalez Araya et al. (2012), whether solely due to predation, lack of settlement, disease or a combination of factors, would be beneficial to the *O. edulis* hatchery process.

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Chapter 5

Monitoring the initial development and progression of *Bonamia ostreae* infection in stocks of the European flat oyster, *Ostrea edulis*, with differing susceptibility to infection

Abstract

Bonamiosis is a lethal disease of *Ostrea edulis*, caused by infection with the protozoan parasite *Bonamia ostreae*. The complete mechanisms of infection of this disease are not yet full understood. The aim of this study was to elucidate the activity of the parasite during the initial stage of infection, the so called "latent period" of infection, which can span a number of weeks post infection and when the parasite cannot be detected within the host. The study took place with stocks of varying susceptibility and where the populations have had varying periods of exposure, from none to several decades. This study looked at the progression of infection within these stocks post-exposure. Three stocks of *O. edulis* with short-term, long-term and no previous exposure to *B. ostreae* were exposed to the pathogen in transmission trials in the laboratory, over a period of 16 weeks. Samples were screened once a week, using heart imprints, PCR analysis and *in-situ* hybridisation (ISH), for prevalence of infection and progression of the infection within the oysters. In Clew Bay and Lough Foyle, both previously exposed stocks when the trial began, the prevalence of infection was higher in the experimental animals compared to the controls, indicating that the addition of *B. ostreae* to the tanks resulted in this increased infection. Loch Ryan, a naïve stock, screened negative for infection throughout the trial, using both heart imprints and PCR analysis of gill and heart tissue, yet *B. ostreae* DNA was detected within the tissue by *in-situ* hybridisation. *B. ostreae* cells quickly entered the host via the gills and digestive tract. *B. ostreae* was observed in all oyster tissues later on in the trial, implying the cells were spreading throughout the oysters. The cells were observed free and intracellularly in haemocytes in the connective tissue but not in the heart. The trial finished before it could be determined if the infection detected by ISH in the Loch Ryan samples could

develop into a viable infection. If so, this would have major ramifications for the current methods used in the diagnosis of *B. ostreae* as neither heart imprints nor PCR analysis detected the parasite during the latent period of the disease.

Introduction

Bonamia ostreae is an intracellular protozoan parasite of the European flat oyster, *Ostrea edulis*. It is the causative agent of the disease bonamiosis, which has caused significant losses amongst *O. edulis* populations worldwide (Lynch et al. 2007). Despite being discovered over 30 years ago (Pichot et al. 1979), several aspects of the disease are not yet fully elucidated, such as the initial progression of *B. ostreae* infection within *O. edulis* tissue. Oysters exposed to the parasite show no clinical sign of infection within their cells for between four weeks and several months following exposure to the parasite, the so-called "latent period" (Tigé & Grizel 1984, Elston et al. 1987, Montes 1991, Culloty & Mulcahy 1996, Culloty & Mulcahy 2001). Thorough examination of tissue sections during this period using the histological- based diagnostic methods failed to detect any early or developmental stages of the parasite (Culloty & Mulcahy 2001).

Montes et al. (1994) concluded, using electron microscopy, that *B. ostreae* enters a flat oyster through the gill epithelia and subsequent to the latent period of approximately ≥ 4 week, *B. ostreae* parasitizes the haemocytes and the cells of the gill (Culloty & Mulcahy 2007). It multiplies by binary fission within the infected haemocytes, eventually rupturing the cells and spreading throughout the tissue (Poder et al. 1983, Culloty & Mulcahy 2007). Eventually the parasite can be observed extracellularly in the tissues, resulting in massive inflammation and the death of its host (Bucke & Feist 1985, Arzul et al. 2009). Closely related species, such as *Bonamia exitiosa* and *Bonamia roughleyi*, have very similar pathologies to *B. ostreae* (Hine 1996, Cochennec-Laureau et al. 2003b), yet neither experience an initial latent period, thus no clear analogy or indication of what may be occurring with *B. ostreae* can be made during this stage.

Three stocks with varying histories of exposure to *B. ostreae* were selected for use in this study to monitor how the disease might progress in different oyster stocks with varying lengths of exposure to *B. ostreae* and possibly show differences in susceptibility. A stock from Clew Bay, located on the west coast of Ireland, was chosen as it has been exposed to the disease for a period of 25 years, since 1988. Though the disease has persisted over this time period, some oysters survive to market size and beyond and it is likely that some individuals have developed some tolerance or resistance to the disease. A stock from Lough Foyle, located on the north coast of Ireland, was selected as it's exposure to the parasite occurred eight years ago, in 2005, possibly an insufficient period of time for the stock to develop any substantial tolerance or resistance to the parasite, as this may take several generations (Culloty et al. 2004). Finally, stock from Loch Ryan, on the west coast of Scotland, was chosen, as this stock is naïve and has never been exposed to *B. ostreae* infection, therefore one should expect they are the most susceptible to the pathogen and progression of infection would develop rapidly.

The study was complicated by the fact that a percentage of both Clew Bay and Lough Foyle oysters were infected with *B. ostreae* before the trial commenced. As a result it was hoped that new infections could be generated in the uninfected cohort by the addition of *B. ostreae* cells to the tanks. These new infections would initially be identified as class 0 infections, progressing to higher classes of infection in the weeks following exposure.

The objectives of this study were to determine the progression of the parasite within *O. edulis,* upon initial infection with the disease and to compare the prevalence and location of the infection between naïve, short-term and long-term exposed oyster

stocks within this time period. Also any differences in host responses to exposure were investigated.

Materials And Methods

Tank trial

Three stocks were selected; Clew Bay, a wild fishery that has been affected by *Bonamia ostreae* since 1988, situated on the west coast of Ireland, Lough Foyle, a wild fishery where *B. ostreae* was first detected in 2005, found on the north coast of Ireland and Loch Ryan, a managed fisheries, unexposed to *B. ostreae,* situated on the west coast of Scotland. Natural settled spat, approximately less than a year old, was sourced from all three stocks. A sample of 60 oysters, from each stock, was screened, using heart imprints and PCR, prior to the trial, to assess and determine prevalence of infection. The oysters were placed in three separate tank systems containing four 400-litre tanks each. 50 oysters were placed in each 400-litre tank, 200 oysters from each stock in total. Oysters were then exposed to infection through the addition of homogenised *Ostrea edulis* tissue, from Lough Foyle, containing *B. ostreae* cells (approx. $1x10^6$), calculated using a haemocytometer, to each system. Tanks were maintained at 15°C and the oysters were fed once per week, 5ml per tank, with Instant Algae shellfish diet 1800®. A 20% water change was carried out every two weeks.

Sampling

Eight oysters (two per tank) were sampled from each system (n=24) at 24 hours, 48 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks and 16 weeks, depending on mortalities (n=304). The trial finished after the 16^{th} week of sampling due to mortalities. Heart imprints were carried out on all oysters. PCR analysis of gill tissue was carried out on all samples (n=304). PCR

analysis of heart tissue was also carried out on all Loch Ryan samples (n=104). *In-situ* hybridisation was carried out on two oysters (n=50), from Clew Bay and Lough Foyle on each sampling date, which were selected based on having no or a low level of infection, possibly indicating a new infection, following exposure in the trial. These oysters had high intensities of infection and were negative by PCR analysis or *vice versa*. *In-situ* hybridisation was carried out on all Loch Ryan samples (n=104).

Processing and examination of ventricular heart imprints

Each oyster was opened, the complete heart removed, placed onto tissue paper and the ventricular heart imprinted onto slides. The imprints were air dried before being fixed in methanol for two minutes and stained with Hemacolour 2 and 3 (Merck)(Culloty et al. 1999). The imprints were examined under light microscopy at 400x magnifications. The intensity of *Bonamia ostreae* infection was determined using the following scale:

Class 0: No *B. ostreae* cells observed.

Class 1: 1–10 *B. ostreae* cells observed.

Class 2: 11–100 *B. ostreae* cells observed.

Class 3: *B. ostreae* cells observed in all fields of vision

Class 4: *B. ostreae* cells observed in all cells (Bachère et al. 1982, Culloty et al. 2004).

DNA isolation and PCR analysis

Pieces of gill tissue (5 mm) were collected from each animal for the Clew Bay, Lough Foyle and Loch Ryan samples. Heart tissue was also collected from the Loch Ryan samples. DNA was extracted from the tissues using the Chelex-100 method

(Walsh et al. 1991, Lynch et al. 2010). The extractions were stored at –20°C. PCR analyses were performed with primers Bo Boas, which amplified 300 bp from the SSU rRNA gene, using the method of Cochennec et al. (2000). All PCR assays included positive controls (*Bonamia ostreae* DNA) and a negative control (double distilled water). The PCR products were run on 2% agarose gel in TE buffer gel (110V and for 40 mins) stained with ethidium bromide (10mg/L). Results were categorised as either positive or negative.

In-situ hybridisation

Tissue sections were placed on silane-prep slides (Sigma Aldrich™). The primers Bo Boas were again chosen (Cochennec et al. 2000) and the probe labelled with digoxigenin, incorporated by PCR. All solutions and buffers used were made up according to the protocol described by Sambrook and Russell (2001) and Lynch et al. (2008) and cover slipped with Eukitt mountant (Sigma-Aldrich™). Results were categorised as either positive or negative. If positive for infection, the area of infection was noted.

Results

Prevalence of Infection

The initial health screening of both Clew Bay and Lough Foyle samples showed the same prevalence of infection, 33% in both stocks with 30% detected using heart imprints and an additional 3% detected using PCR analysis. Loch Ryan samples showed no infection when screened using both heart imprints and PCR analysis of gill tissue (Fig.1).

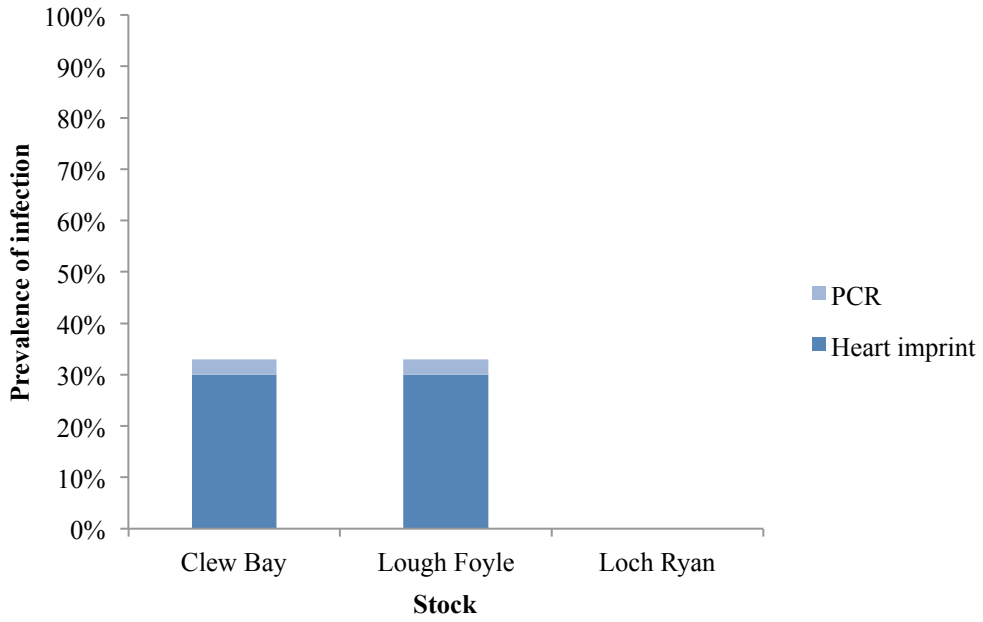


Fig.1. Initial prevalence of infection (%) by *Bonamia ostreae* in all three trial stocks prior to the trial commencing.

Heart imprints

A number of oysters from Clew Bay (21%), Lough Foyle (52%) and Loch Ryan (48%) died during the study and could not be screened for infection, due to degradation of the tissues. Clew Bay oysters had a prevalence of infection of 12.5% when oysters were screened 24 hrs post exposure. The prevalence of infection increased over the course of the trial peaking at 50% in week 8 before declining again to 0% in week 9 (Fig. 2). 27% (54/200) of the stock survived to the end of the trial, up to week 16.

Lough Foyle's prevalence of infection was 0% after 24 hrs, rising to 12.5% after 48 hrs. Prevalence of infection in Lough Foyle followed a similar pattern to that of Clew Bay with prevalence increasing to a prevalence of 50% in week 2 (Fig. 2). Complete mortality of the Lough Foyle oysters occurred during week 10.

B. ostreae was not detected in the initial Loch Ryan sample and no subsequent infection was found by heart imprint in the Loch Ryan samples (n=104) over the course of the trial. Complete mortality of the Loch Ryan oysters occurred during week 16.

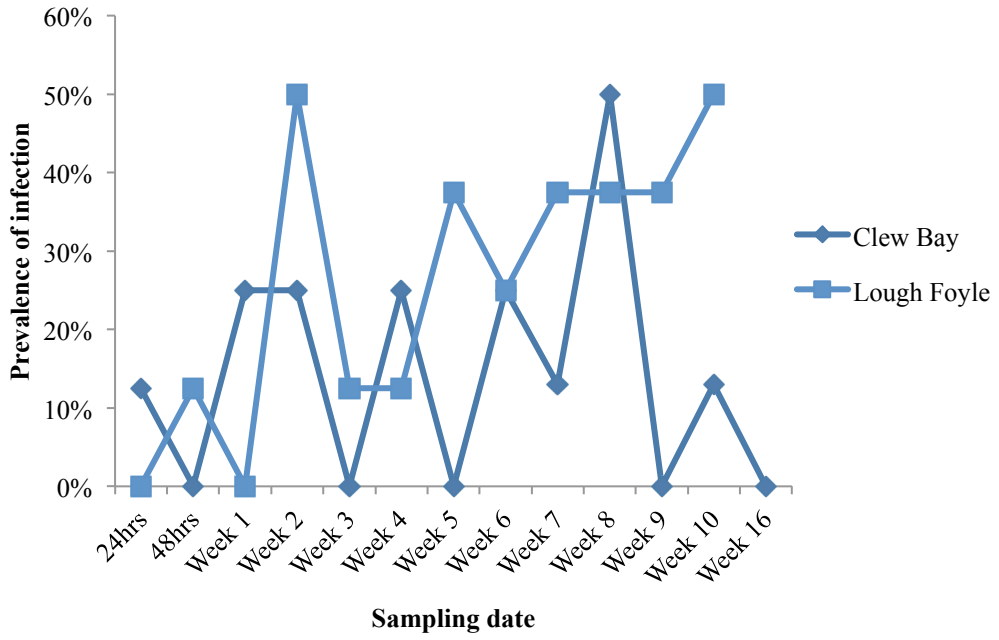


Fig.2**.** Prevalence of infection of stocks per sampling time using heart imprints.

No class 4 infections were observed in the Clew Bay samples over the course of the study. The highest infection observed was class 3 infections. By week 8, 25% of all the oysters sampled had class 3 infections. Little to no infection was noted in the oysters sampled after this week (Fig. 3). As with Clew Bay, no class 4 infections were observed in the Lough Foyle samples over the course of the study. Class 3 infections dominated from week 5 until week 10. As previously stated, full mortality occurred in the samples during week 10 (Fig. 4).

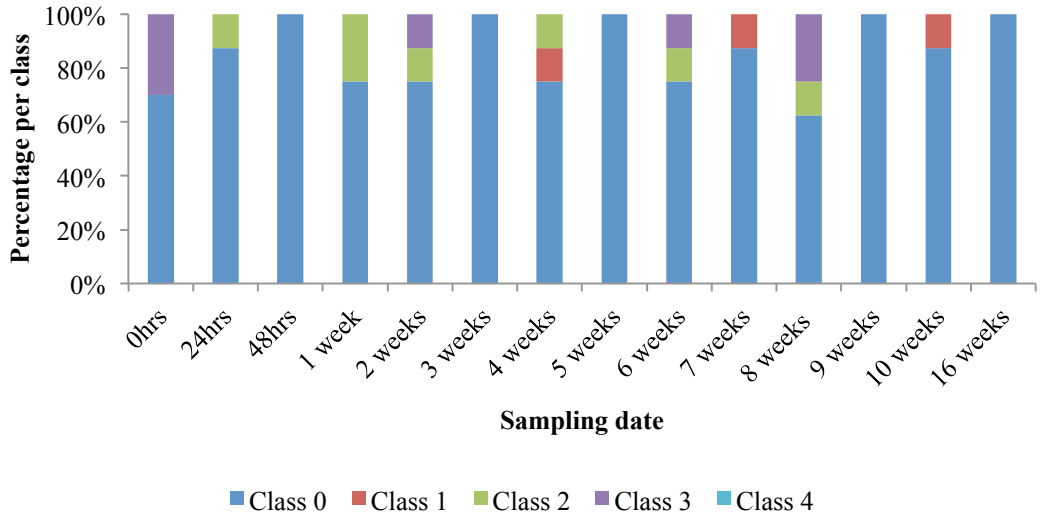


Fig.3. Intensity of infection in Clew Bay oysters over the 16-week study period.

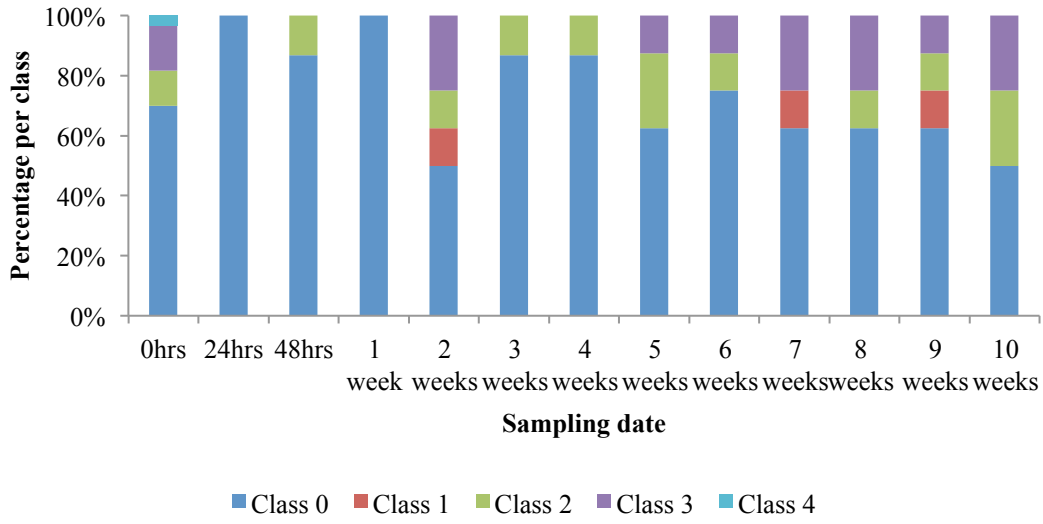


Fig.4. Intensity of infection in Lough Foyle oysters over the 10-week study period.

No irregularities were noted when the *Bonamia ostreae* cells were observed in the heart imprints of both stocks over the course of the study. In both Clew Bay and Lough Foyle, *B. ostreae* cells were observed both extracellular and intracellular (Fig. 5). Only uninucleate and binucleated cells were observed in both stocks, plasmodia were absent (Fig. 6).

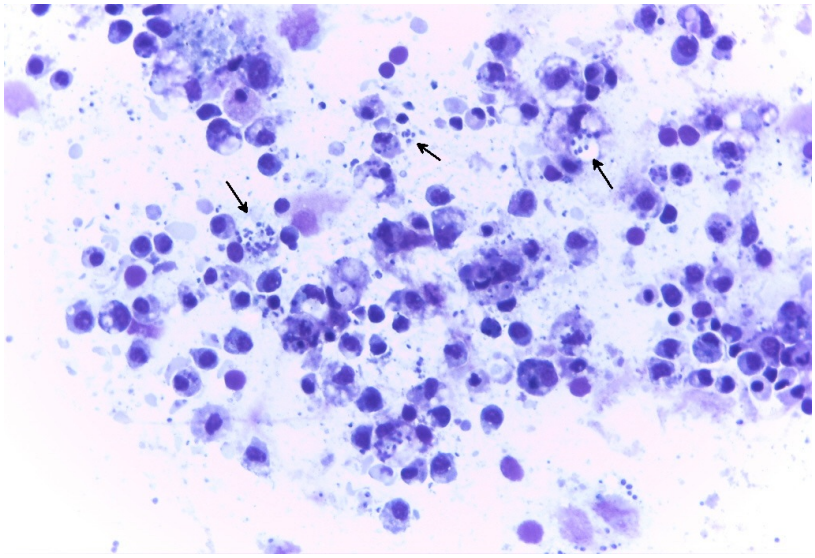


Fig. 5. Heart imprints from Clew Bay, displaying hemocytes with arrows indicating extracellular and intracellular *Bonamia ostreae* cells.

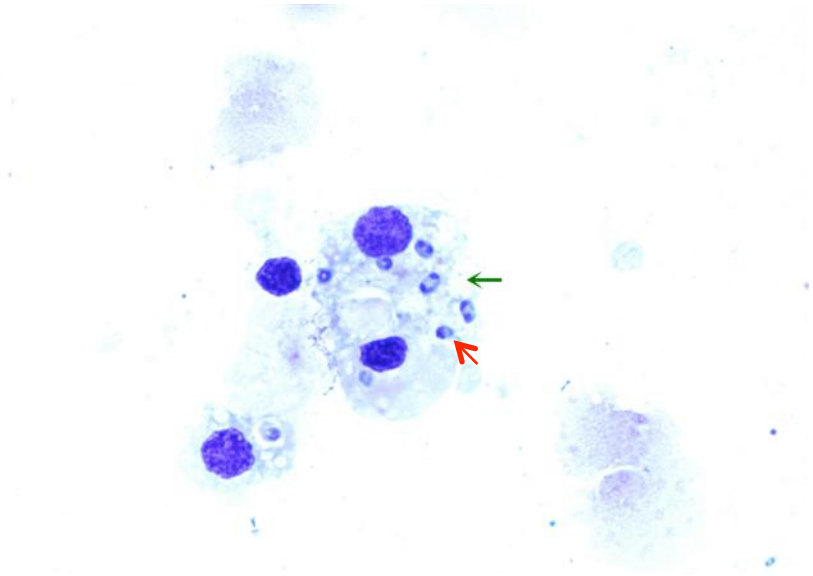


Fig. 6. Heart imprint displaying uninucleate (red arrow) and binucleate (black arrow) *Bonamia ostreae* cells.

PCR analysis

No infection was found by PCR analysis of heart and gill tissue in the Loch Ryan samples over the course of the trial. In both Clew Bay and Lough Foyle, some samples found to be negative by heart imprint were confirmed to be positive when PCR analysis was carried out, and *vice versa*, thus the PCR analysis provides a marginally different perspective on the prevalence of infection.

Again in both stocks, the prevalence of infection varied over the study period. In contrast to the heart imprint results, PCR analysis showed Clew Bay oysters had the highest prevalence of infection in week 6, initially, before peaking again in week 9 (Fig. 7), while Lough Foyle oysters demonstrated a peak in infection in week 2 using heart imprints, they demonstrated a slow incline in infection during the initial weeks using PCR analysis (Fig. 8).

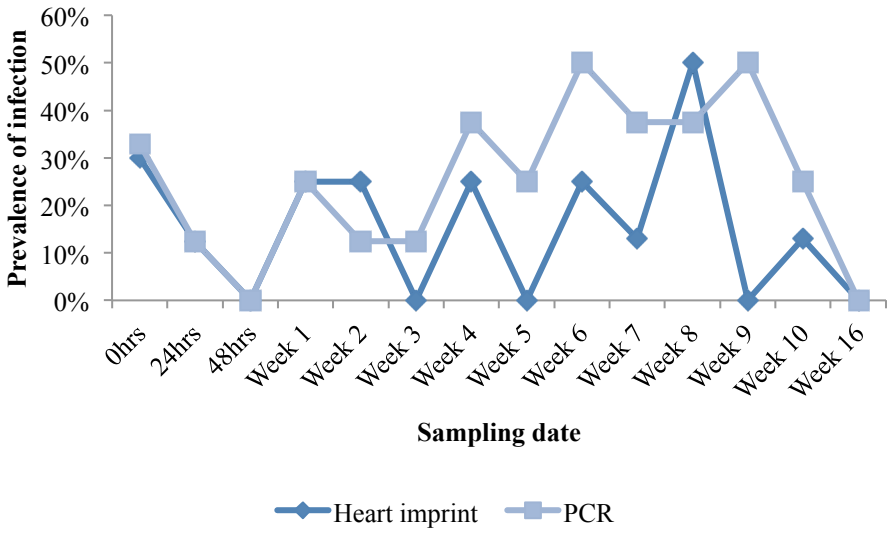


Fig.7. Detected prevalence of infection of *Ostrea edulis* in Clew Bay per sampling time using heart imprints and PCR analysis.

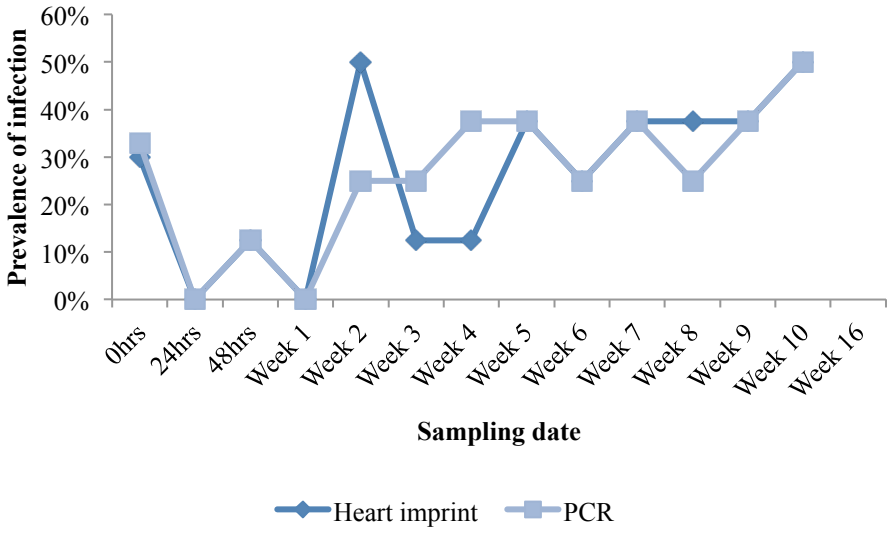


Fig.8. Detected prevalence of infection of *Ostrea edulis* in Lough Foyle per sampling time using heart imprints and PCR analysis.

In-situ hybridisation (ISH)

Following screening using heart imprints and PCR analysis, two animals from each stock and from each sampling date were subsequently screened using ISH to try to locate the parasite within the oyster's tissue. For Clew Bay and Lough Ryan oysters that were known to be infected when brought into the laboratory, animals diagnosed as negative for infection or that had a low level of infection were generally chosen for this screening as these animals were thought to be the most likely individuals from each stock to have been exposed initially to the parasite in the laboratory trial and in addition, were more likely to be individuals going through the latent stage.

Twenty-six Clew Bay oysters were chosen over the study period to monitor progression of *B. ostreae* cells within the tissues using *in-situ* hybridisation, 19 of which tested positive for *B. ostreae,* using heart and/or PCR analysis. 38.5% (10/26) of samples were detected positive by heart imprint while 69.2% (18/26) were positive by PCR analysis. Positive staining, using ISH, was observed in 53.8% (14/26) of these individuals (Table 1) (Fig. 9). 57% of all infection observed was in the connective tissue while 29% was observed in the gill filaments and 14% in the digestive system. There appeared to be no progression of infection within the tissues as the trial continued, similar tissues were found infected at the end of the trial as at the beginning. Of the 14 positive Clew Bay ISH's, 79% were also detected by PCR analysis while only 36% detected by heart imprint. 14% of the positive ISH samples were not detected by either heart imprint or PCR analysis. The higher the class of infection did not have an impact on whether the parasite was detected by ISH or not. In class 0 infections confirmed positive by ISH, the animals considered most likely to be in the latent stage, 57% (9/14), 44% of oysters demonstrated reactivity in the connective tissue and another 44% were in the gill tissue while 12% were in the

digestive tract. Heavy, class 3 infections were observed solely in the connective tissue (Table 1).

Table 1. Results of screening of two animals from each stock, Clew Bay and Lough Foyle, per sampling date, using heart imprints, PCR and subsequent screening to monitor progression of infection within the host, using *in-situ* hybridisation.

	Clew Bay										Lough Foyle									
Time	Class of infection		Heart Imprint		PCR		ISH		Infected tissue	Infected tissue	Class of infection		Heart Imprint		PCR		ISH		Infected tissue	Infected tissue
24hrs	2	0	+	–	+	–	+	–	Connective	–	0	0	–	–	–	–	–	–	–	–
48hrs	0	0	–	–	–	–	+	–	Connective	–	2	0	+	–	+	–	+	+	Gill	Connective
1 week	3	0	+	–	+	–	–	–	Connective	–	0	0	–	–	–	–	–	–	–	–
2 weeks	2	3	+	+	+	–	–	+	–	Connective	1	2	+	+	–	+	+	–	Gill	–
3 weeks	0	0	–	–	+	–	–	–	–	–	2	0	+	–	+	+	+	+	Connective	Gill
4 weeks	1	0	+	0	+	+	+	+	Digestive tract	Connective	2	0	+	–	+	+	–	–	–	–
5 weeks	0	0	–	–	+	+	+	–	Connective	–	2	3	+	+	+	+	–	–	–	–
6 weeks	2	3	+	+	+	+	+	–	Connective	–	2	0	+	–	+	+	+	–	Gill	–
7 weeks	0	0	–	–	+	+	+	+	Gill	Gill	1	3	+	+	+	+	–	+	–	Connective
8 weeks	1	2	+	+	+	+	–	+	–	Connective	2	3	+	+	+	+	–	–	–	–
9 weeks	0	0	–	–	+	+	+	+	Gill	Digestive tract	1	2	+	+	+	+	–	+	Gill	–
10 weeks	1	0	+	–	+	+	–	+	–	Gill	2	3	+	+	+	+	+	+	Connective	Digestive tract
16 weeks	0	0	–	–	–	–	+	–	Connective	–	0	0	-	-	-	-	-	-	–	–
Total	-		**10/26 (39%)**		**18/26 (69%)**		**14/26 (54%)**		–		-		**16/24 (67%)**		**18/24 (75%)**		**10/24 (39%)**		–	

(a) (b)

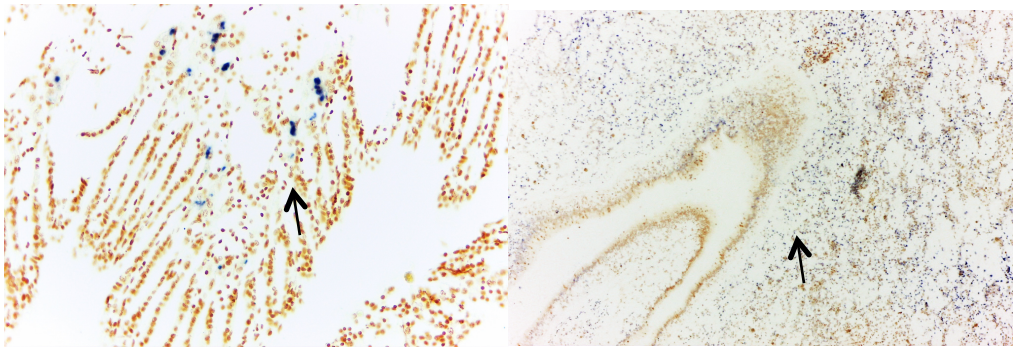


Fig.9. ISH reactivity of *Bonamia ostreae* cells (dark blue, arrows) in (a) gill tissue of Clew Bay *Ostrea edulis* 48 hours of post exposure to *B. ostreae,* which was negative by heart imprint and PCR analysis, (b) connective tissues of Clew Bay *O. edulis* 2 weeks of post exposure to *B. ostreae,* which was positive by heart imprint but negative by PCR analysis.

Twenty-four Lough Foyle oysters, 19 of which tested positive for *B. ostreae,* using heart and/or PCR analysis, were screened using *in-situ* hybridisation. 66.7% (16/24) of the oysters were detected positive by heart imprint, while 75% (18/24) were positive by PCR analysis. A positive signal using ISH was observed in 41.7% (10/24) of these samples (Table 1) (Fig. 10). 50% of all infection observed was in the gill filaments while 40% was observed in the connective tissue and 10% in the digestive system. As with Clew Bay, there appeared to be no progression of infection within the tissues as the trial continued. Of the 10 positive Lough Foyle ISH's, 80% were also detected by PCR analysis and 80% detected by heart imprint. 10% of the positive ISH samples were not detected by either heart imprint or PCR analysis. Again, the higher the class of infection did not have an impact on whether the parasite was detected by ISH or not. In the 20% (2/10) of oysters with class 0 infections confirmed positive by ISH, the animals most likely to be in the latent stage, 50% were in the connective tissue while the other 50% were in the gill tissue, very similar to the results found in Clew Bay. Heavy, class 3 infections were observed mainly in the connective tissue (Table 1).

(a) (b)

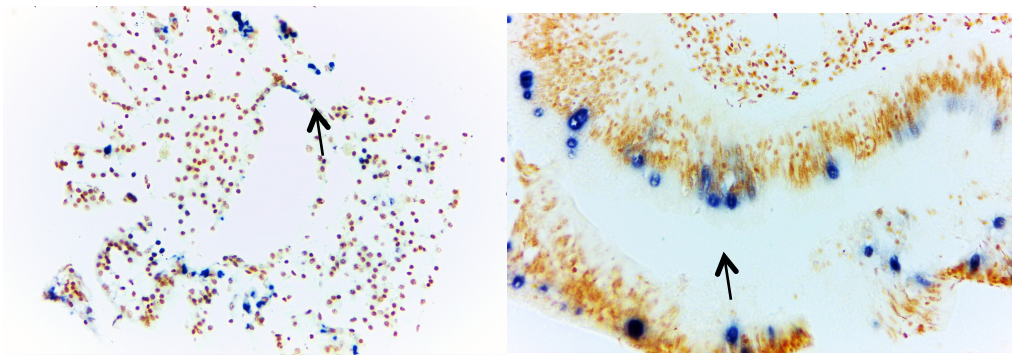


Fig.10. ISH reactivity of *Bonamia ostreae* cells (dark blue, arrows) in (a) the connective tissue of Lough Foyle *Ostrea edulis* 48 hours of post exposure to *B. ostreae*, which was positive by heart imprint and PCR analysis (b) the digestive tissues of Lough Foyle *O. edulis* 10 weeks of exposure to *B. ostreae*, which was positive by heart imprint and PCR analysis.

All 104 Loch Ryan oysters, which tested negative for *B. ostreae* using heart imprints and PCR analysis, were screened using *in-situ* hybridisation. Positive signals were observed in 51% of these oysters (53/104) (Table 2). A positive signal, using ISH, was observed in several tissues 24 hours after introduction of the parasitic cells to the tank system (Fig. 11). Again at 48 hours positive signals were observed in several tissues (Fig. 11). Positive signals continued to be observed up until the 16 week of the study. In some samples there was evidence of the cytoplasm of the *O. edulis* blood cells staining positive.

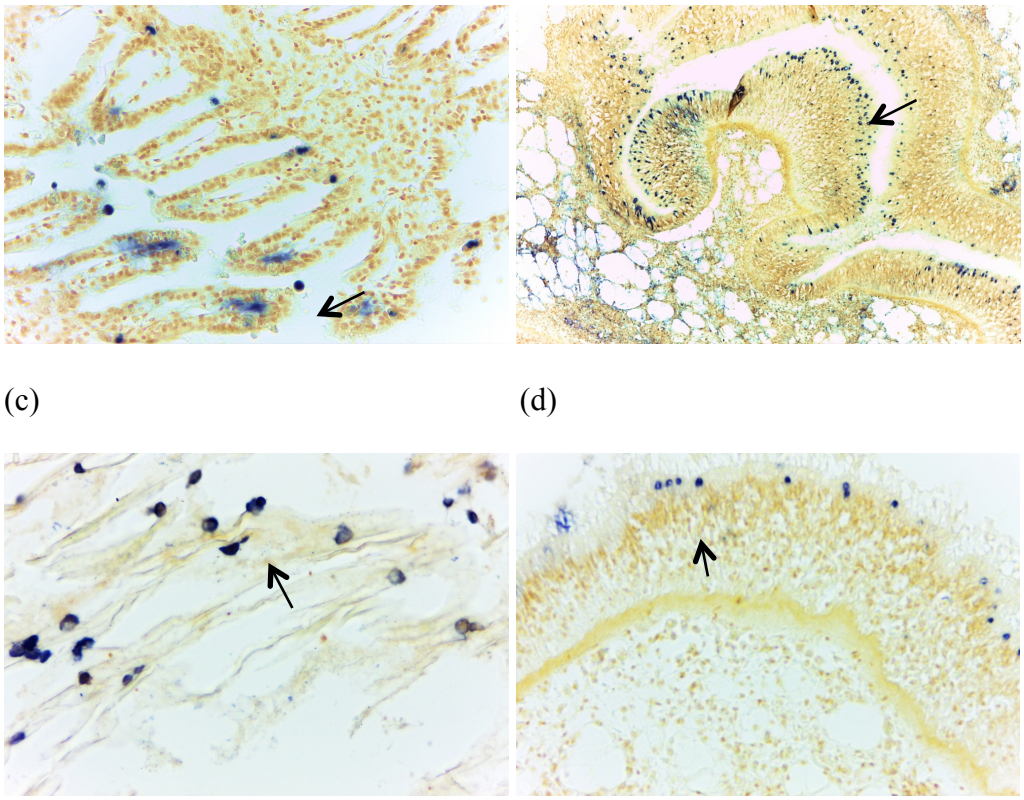
45% of all infections observed were in the gill filaments while 21% were observed in the connective tissue, 4% in the mantle tissue, 6% in the digestive tract and 23% in all tissues. The majority if infections at the start of the study were observed in the gill and digestive tissue but by the completion of the trial many samples displayed

infection throughout the tissues (Table 2). Unlike Clew Bay and Lough Foyle, progression of infection within the tissues was observed as the trial continued in these class 0, possible latent, infections.

Table 2. Results of screening of eight animals from Loch Ryan, per sampling date, using heart imprints, PCR and subsequently screened to monitor progression of infection within the host using *in-situ* hybridisation.

	Loch Ryan											
Time	**Class of infection**	**Heart Imprint**	**PCR**	**ISH**	**Infected tissue**	**Infected tissue**	**Infected tissue**	**Infected tissue**	**Infected tissue**	**Infected tissue**	**Infected tissue**	**Infected tissue**
24hrs	0	0/8	0/8	4/8	Gill	Gill	Gill	Gill	–	–	–	–
48hrs	0	0/8	0/8	3/8	Gill	Digestive tract	All	–	–	–	–	–
1 week	0	0/8	0/8	5/8	Gill	Gill	Connective	Connective	Gill	–	–	–
2 weeks	0	0/8	0/8	5/8	Connective	Connective	Connective	Gill	Gill	–	–	–
3 weeks	0	0/8	0/8	6/8	Gill	Gill	Gill	Gill	Digestive tract	Connective	–	–
4 weeks	0	0/8	0/8	5/8	Gill	Gill	Connective	all	All	–	–	–
5 weeks	0	0/8	0/8	2/8	Gill	All	–	–	–	–	–	–
6 weeks	0	0/8	0/8	5/8	Gill	Gill	Gill	Mantle	Digestive tract	–	–	–
7 weeks	0	0/8	0/8	4/8	Gill	Connective	Connective	All	–	–	–	–
8 weeks	0	0/8	0/8	3/8	Gill	Connective	Digestive tract	–	–	–	–	–
9 weeks	0	0/8	0/8	2/8	all	All	–	–	–	–	–	–
10 weeks	0	0/8	0/8	5/8	Gill	Mantle	All	All	All	–	–	–
16 weeks	0	0/8	0/8	4/8	Gill	Connective	All	All	–	–	–	–
Total	-	**0/104 (0%)**	**0/104 (0%)**	**53/104 (51%)**	–							

(a) (b)



(c) (d)

Fig. 11. ISH reactivity of *B. ostreae* cells (dark blue, arrows) in the tissue of Loch Ryan *O. edulis* (a) 24 hours post exposure to *B. ostreae* in the gill tissue (b) 48 hours post exposure to *B. ostreae* in the digestive and underlying tissue (c) 1 week post exposure to *B. ostreae* in the connective tissue (d) 10 weeks post exposure to *B. ostreae* in the mantle tissue.

Discussion

In this study, the progression of the parasite within *Ostrea edulis,* following exposure in the laboratory, was monitored and the prevalence and location of the infection between naïve, short and long term infected oyster stocks was compared. Both Clew Bay and Lough Foyle showed an increase in prevalence of infection over the study period, while Loch Ryan, a stock naïve to *Bonamia ostreae* infection, screened negative for infection using both heart imprints and PCR, the OIE recommended diagnostic methods (OIE 2012). Clew Bay and Lough Foyle displayed clinical signs of infection within the first week post exposure, most likely due to natural exposure to the parasite in the wild before commencement of the trial though signs of new, latent infections were also present, infections that were determined to be class 0 by heart imprint yet clearly displayed *B. ostreae* cells within their tissues when examined by ISH.

Lough Foyle oysters suffered mortalities after week 10, during which they showed a prevalence of infection of 50%, a high percentage of which were class 3 infections, implying that these mortalities were probably due to the infection. All the Clew Bay oysters sampled in week 16 were uninfected, while the two previous sampling weeks displayed little to no infection. This may indicate that a small proportion of Clew Bay stock may have some tolerance to *B. ostreae* while Lough Foyle, a recently exposed stock, has yet to develop any or is in the early stages of development. Observations of *B. ostreae* in the digestive system of oysters from all three stocks may indicate that the animals were ridding themselves of the parasite. A greater number of the Clew Bay stock was found to have *B. ostreae* cells in their digestive system compared to the Lough Foyle stock sampled, supporting the theory that Clew Bay has some establish tolerance to the disease or perhaps are able to eliminate more

parasites from the body via apoptosis. The Clew Bay oysters with cells in their digestive tracts were class 0 to class 1 infections, though the single Lough Foyle with cells in the digestive tract was a class 3. It is also possible that these animals were picking up cells through ingestion.

Montes et al. (1994) in an electron microscopy study, found that *B. ostreae* entered the flat oyster through the gill epithelia, which would be supported by the results observed in this study. van Banning (1990) hypothesised that the initial stages of bonamiosis occur in the ovarian tissues, yet in this study no infection was observed by ISH in gonadal tissue. The activity of the *B. ostreae* cells during this latent period still remains fairly obscure.

When screened by ISH, *B. ostreae* cells were confirmed within the tissue of the Loch Ryan stock. ISH was used alongside heart imprints and PCR analysis as *B. ostreae* can be visualised in the tissue using this technique, thus the progress of the parasite can be traced in the animal. ISH possesses a similar or higher sensitivity to that of PCR, allowing for the detection of low intensities of infection (Carnegie et al. 2003, Diggles et al. 2003, Lynch et al. 2008, Culloty & Mulcahy 2007). This level of sensitivity enables the detection of *B. ostreae* during its initial infection (Culloty & Mulcahy 2007). It is not possible to detect the parasite in the host's tissues with heart imprints during this initial period (Poder et al. 1982) and if infection is focal within an animal, PCR analysis can fail to detect the infection as only a small section of tissue is used in this diagnostic method (Diggles et al. 2003, Lynch et al. 2008).

Small proportions of the Clew Bay samples and Lough Foyle samples screened, using all three diagnostic methods, screened positive for infection using ISH yet were negative using both heart imprints and PCR analysis. These may be new, latent infections in both stocks caused by the addition of *B. ostreae* cells to the water.

These results support the theory that a new infection, in its latent stage, may be unrecognisable by cytology and too localised for PCR analysis. Again in the Clew Bay samples, the majority of all positive ISH samples were also confirmed by PCR analysis while only a third were confirmed by heart imprint, indicating that as the disease progress it may become more widespread throughout the tissues allowing for detection using PCR analysis before finally progressing to a full-scale clinical infection, whereby it can be detected by heart imprint. It is possible that if the Loch Ryan oysters had survived longer, the cells observed would have developed into a clinical infection, while some of the Clew Bay and Lough Foyle class 0 individuals may have been able to eliminate the parasite.

B. ostreae was observed in all tissues, with the exception of the haemocytes of the heart, throughout the trial in the Loch Ryan stock, showing the parasite was spreading throughout the oysters. It was observed throughout the gills, mantle, digestive tract and connective tissue**.** The fact that it was observed so extensively in tissues, such as gill and connective, yet not in the heart imprints is a common occurrence during the latent stage. Perhaps *B. ostreae* was present in the heart but during this latent stage the parasite is in a form not yet identifiable by cytology. *B. ostreae* cells have been observed to take several forms including uninucleate, binucleate and plasmodial forms thus it is feasible another form exists. A new stage of *Bonamia exitiosa*, which was previously considered morphologically similar to *B. ostreae,* has been discovered by Hine et al. (2001). The stage was found to have a large vacuole with a poorly delineated nucleus.

As previously stated, clinical bonamiosis may have developed in the Loch Ryan stock if observed for a longer period of time. Previous studies have reported it takes periods of 3 to 6 months for clinical signs of infection to occur (Poder et al. 1982,

Tigé & Grizel 1984, Grizel 1985, Elston et al. 1986, Montes 1991, Hervio et al. 1995, Culloty & Mulcahy 1996, Culloty et al. 2004, Lallias et al. 2008). Perhaps a specific trigger, such as an environmental change, or the life cycle progressing to the next stage under a particular set of circumstances, is required to end the latent/initial infection period as the transmission of the disease also depends on environmental factors such as temperature, salinity or physical manipulation (Lallias et al. 2008) or perhaps not all latent/initial infections progress into full, clinical infections.

It is also possible that Loch Ryan stock can become infected with *B. ostreae* but are able to eliminate the disease, as previous trials carried out in UCC have had limited to no success in infecting the stock. In a previous study of Loch Ryan oyster susceptibility, 540 Loch Ryan oysters of three different ages, held at *B. ostreae* infected Rossmore, Cork for four months were free of infection when screened using heart imprints, while *B. ostreae* DNA was detected in the PCR screening of two oysters in the final screening. Loch Ryan oysters were again deployed to Rossmore with 60 oysters sampled on day 1, week 1, week 4 and week 7 of the trial. *B. ostreae* was not detected in any of the 240 oysters screened using heart imprints and PCR (Lynch et al, unpublished data). Although cells resembling *B. ostreae* stained positive in the ISH of the Loch Ryan samples, there was also evidence of the cytoplasm of the *O. edulis* blood cells staining positive also which might indicate that traces of degraded or digested parasite DNA was being detected. Compared to the Clew Bay and Lough Foyle stocks, Loch Ryan is a relatively slow growing stock, which may contribute somewhat to the lack of *B. ostreae* development within it.

It is feasible though that, as the mortality rate was 100% after week 16 in this study, the Loch Ryan oysters are succumbing to infection before the disease has emerged

from its latent stage, the severe reaction of a completely naïve stock, whereby the stress of exposure to a new pathogen caused complete mortality, or it may be stress related mortality from holding conditions. Lacoste et al. (2001) found mortality levels increased in stressed *Crassostrea gigas* exposed to *Vibrio splendidus* due to increased levels of neuroendocrine stress response hormones being produced in the animals. These hormones redirect energy to specific physiological functions, which may weaken the animal's immune defenses against a pre-existing threat such as the presence of pathogen.

A very low number of *B. ostreae* cells were added to the tank, as a dose of 50,000 to 80,000 parasites per oyster is recommended (Martin et al. 1993, Hervio et al. 1995). A low dose was used to replicate the natural exposure of oysters in the wild to the disease. Due to seawater dilution, *O. edulis* would rarely, if ever, be exposed to high doses of the parasite. Also, as Loch Ryan is a naïve stock, exposure to high doses of *B. ostreae* may have resulted in rapid mortalities. However, the fact that positive ISH results were obtained in Loch Ryan stock over the sampling period indicates that *B. ostreae* cells were replicating in the system.

This study indicates that even with exposure to a low number of cells, *O. edulis* still has to capability to become infected with *B. ostreae* or, at least, to carry the pathogen within its tissues. The parasite enters the host through the gill, epithelium and digestive system. The study also demonstrates the risk of present diagnostic methods for *B. ostreae.* Though the Loch Ryan animals were carrying the parasite, both heart imprints and PCR failed to detect it. This may have major implications for the movement of stock. A stock that is confirmed negative by both methods may be uninfected or simply experiencing the latent period of the disease. The movement of such a stock would allow for further transmission of bonamiosis. Additional research

is required to see if the parasite observed by ISH in Loch Ryan tissue can indeed develop in to a viable and/or transmittable infection and if it cannot, what biochemical reactions are occurring within this oyster stock to prevent this. Histology may also be carried out on the Loch Ryan samples to obtain if *B. ostreae* cells can be observed in the tissue or if not, if there are any unusual features present in the tissues. It would also be of interest to expose a different naïve oyster stock to *B. ostreae* and compare the performance of that stock with the Loch Ryan stock. Additional research is also required into the Clew Bay stock to ascertain whether some oysters have developed a tolerance to the disease or whether they will all eventually succumb to infection post exposure.

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Chapter 6

Discussion and Conclusions

Bonamia ostreae has had a devastating impact on European flat oyster populations since its introduction into Europe in the 1970's. As previously stated, mortality rates of over 90% were observed when the parasite first invaded some populations (McArdle et al. 1991), causing production of *Ostrea edulis* to plummet from 30,000 tons in the 1960s to the 6,000 tons produced today (Lallias et al. 2008). As a result, the majority of oyster fisheries turned to the production of the Pacific oyster, *Crassostrea gigas*. France, once a major producer of European flat oysters, now produces approximately 120,000 tons of *C. gigas* and only 1,500 tons of *O. edulis* annually (Buestel et al. 2009). The affects of *B. ostreae* on stocks and the shift in focus by the industry to the aquaculture of *C. gigas* have left our native oyster species in critical need of conservation. The continual summer mortalities observed in *C. gigas* stocks throughout Europe (Berthelin et al. 2000) have renewed the industry's interest in the improvement of present *O. edulis* stocks and the possible creation of *B. ostreae* resistant stocks. In order to achieve any success in the conservation of *O. edulis* though, it is essential that we first understand the life cycle and complete means of transmission of the parasite in addition to the interactions of the disease with it's host, both short and long-term, as further spread of the disease worldwide would be profoundly detrimental to the species and proper and comprehensive management is fundamental to the recovery of any species. The objectives outlined in Chapter 1, created with the aim of addressing the issues of the life cycle and disease-host interaction, will be reviewed to determine if they were indeed met and if so, what conclusions can be drawn from the results.

Objective

1. To compare the effectiveness and efficiency of the most commonly used screening tools for the diagnosis of *Bonamia ostreae* in order to establish that these tests are indeed predominately adequate at detecting the disease.

Overall the findings demonstrated that none of the presently used tests are 100% accurate in detecting the disease. These findings would indicate that at least two methods should be used for the accurate detection of the disease within a sample - a microscopy based technique for visualisation of the parasite and a molecular based technique, or moreover, a new form of screening should be devised. Most recently a new PCR analysis was devised using newly designed primers. The test showed high sensitivity yet false positives, again, appeared to be a problem, as with other PCRs (Ramilo et al. 2013). A method similar to ISH that combines the two techniques, traditional and molecular, but with greater ease and less cost appears to be the best solution as the one major limitation to molecular techniques is the occurrence of false positives, with the detection of parasite DNA but not a viable infection - the ability to visualise any infected tissue would eliminate this problem.

The study also found some discrepancies in the results achieved by each of the three laboratories using the same molecular screening methods, indicating that the varied protocols and equipment used by each laboratory was the main issue. Having the OIE introduce a standardised, fixed protocol for each molecular based method can easily rectify this problem. Also equipment used in the methods could be standardised but this would be costly and time consuming. The need for a comprehensive and standardised screening method is an important one in the prevention of the spread of *Bonamia ostreae.* False negatives can allow for the

movement of infected oysters into uninfected zones, while false positives can enable the declaration of an area as a *B. ostreae* infected zone and allow for accidental contamination of the population with the disease through importation of infected seed/spat.

Objective

2. To determine whether exposure to *Bonamia ostreae* over different periods of time in wild, unmanaged flat oyster stocks would result in changes in the prevalence of the disease in these stocks and what contribution environmental and stocking factors would have towards this.

The length of exposure to *Bonamia ostreae* appeared to have little impact on the overall prevalence of infection within a stock. On closer inspection, environmental factors and population densities influence prevalence of infection. It appears that several elements contribute to the survival of the disease within a stock, some of which are beyond the control of husbandry techniques, such as increasing temperatures, while others, such as the effects of low salinity or high density on prevalence, can be worked into the management of stocks. The key point from this study appears to be that management of infected stock is extremely important. Clew Bay, after such a long period of exposure, should be demonstrating some tolerance to the disease but as no management or intervention is in place, in terms of breeding and husbandry, the disease still remains a major issue in terms of low stock numbers. A lack of husbandry management allows prevalences to remain high while no breeding management slows down or prevents the development of a tolerant population within the stock. Emphasis should be placed on establishing a breeding

broodstock that have a higher tolerance to *B. ostreae* from survivors located within the populations.

Objective

3. To determine whether different stocks of *Ostrea edulis* from within a region can be used to restock areas within that region affected by *Bonamia ostreae* infection.

Bonamia ostreae infection appeared not to be a major influence on the survival and growth of various spat stocks in Lake Grevelingen as the Lake Grevelingen spat, which demonstrated the highest overall prevalence of infection, had the highest survival rate and greatest growth rate. It appears local conditions are the main influence on the growth and survival of stocks. These factors can be very localised as the Oosterschelde spat, which came from a stock located beside Lake Grevelingen, failed to perform as well as the Lake Grevelingen spat. The results of this study indicate that, although the Lake Grevelingen, Oosterschelde and Danish stocks come from similar regions and have possibly very similar population genetics, only spat from local oyster populations can be used to restock an area. Many environmental factors, such as temperature, salinity and water chemistry, may be crucial to the development of spat. Further research is needed into this area. The key point of this study is that non-native populations may not be used to stock different areas even within similar regions thus when populations become dangerously low within an area, recovery, using different spat stocks, may not be possible. This highlights the great need to protect already present populations and not rely on restocking for the future of *Ostrea edulis.*

Objective

4. To establish if species of macroinvertebrates found in flat oyster beds or sediment from an infected bed may act as reservoirs or incidental carriers of *Bonamia ostreae.*

In this study, *Bonamia ostreae* DNA was not detected in the sediment by PCR analysis and was not successfully transmitted from exposed sediment to naïve oysters. This would indicate several options; that *B. ostreae* does not have a spore life stage and during this external stage it may only survive in the water column and not in sediment, that no *B. ostreae* was present in the samples used or that the detection method were not sufficient. A study needs to be carried out to establish how long the external life stage of *B. ostreae* remains infectious as uninfected oyster beds can be found within close distance of infected beds, as seen with the uninfected Kilkieran Bay which is located less than 30 km from the infected Galway Bay.

During the screening of macroinvertebrate species, 5 *Mytilus edulis* samples, from October 2010, were found to be positive for *B. ostreae.* However, screening of a further 335 *Mytilus* spp. samples from Europe failed to detect the parasite. As previously stated there was a spike in *Ostrea edulis* mortalities due to *B. ostreae* when the *B. ostreae* positive mussels were sampled thus these animals would have been filtering large amounts of seawater containing large quantities of *B. ostreae* cells which had been released from dying or moribund oysters. Due to the fact that mussels are frequently farmed alongside *O. edulis* beds the discovery of *B. ostreae* in mussels raises some very important issues that need to be addressed to prevent the possible further spread of the parasite through exportation and importation of mussel consignments. Transmission trials need to be carried out to determine if the parasite

continues to be infectious once with *M. edulis*. Further screening of *M. edulis* samples from an area with an *O. edulis* stock experiencing *B. ostreae* induced mass mortality would concluded whether exposure to large quantities of *B. ostreae* cells is required to infect the species or whether, simply, a small number of *M. edulis* are susceptible to the disease. In conclusion this new information on a potential *B. ostreae* life cycle or potential vector highlights the extreme caution required when moving equipment, invertebrates and seawater from infected to uninfected areas as our understanding of how *B. ostreae* can be spread may be very limited.

Objective

5. To ascertain if brooding larvae infected with *Bonamia ostreae* acquired the infection solely from parents or if the source of infection is environmental.

In this study, four different samples of larvae tested positive for the presence of *Bonamia ostreae* DNA yet none of these larvae had *B. ostreae* infected parents. Additionally, infected parents had uninfected larvae. These results indicate that larvae are able to acquire the pathogen from environmental sources. These findings may have implications for the breeding of *Ostrea edulis* in infected areas. Further research is required to know if the disease contributes to larval mortality and if not could these surviving oysters then be considered to be resistant/tolerant to the parasite. In addition, modelling of larval movement in particular areas where *B. ostreae* is present would give an indication of the potential spread of the disease via this method.

Objective

6. To determine the progression of *Bonamia ostreae* within *Ostrea edulis,* from three different stocks with different exposures to the disease, upon initial infection with the parasite and to compare the prevalence and intensity of *B. ostreae* infection between the three stocks within this period.

In this study, both Clew Bay and Lough Foyle showed an expected increase in infection over the study period, while Loch Ryan screened negative for infection using both heart imprints and PCR analysis. The survival of the Clew Bay oysters after completion of the trial and the lack of infection present by week 16 indicates that some tolerance to the disease may exist within the stock. The complete mortality after week 10 and the high prevalence of infection during that week indicates that the Lough Foyle stock has yet to develop any clear tolerance to the disease. Class 0 infections in both were only found in gill and connective tissue in Clew Bay and Lough Foyle while Loch Ryan which were all class 0 infections, showed progression of the disease throughout the tissue. No progression of infection in the tissues was noted either over the course of the study in the Clew Bay and Lough Foyle stocks, with infection continuing to be found in similar tissue at the end of the trial, as at the start. These result reiterates the findings of chapter 2 - proper management of the Clew Bay stocks could allow for the development of a tolerant stock and in turn increase the low populations currently present in the bay, as at present with no management tolerant oysters are being fished out or cross fertilising with susceptible oysters.

The Loch Ryan stock was interesting, as although *Bonamia ostreae* infection was noted within 24 hours of exposure using ISH, no infection was confirmed using heart

imprints or PCR analysis throughout the study. This may indicate that heart imprints and PCR, the two most commonly used methods of detection, may not be adequately sensitive enough to detect newly acquired infections, a clear issue when screening supposedly naïve stocks. Additionally, research is required to see if the parasite observed by ISH in Loch Ryan tissue is a viable infection or whether these oysters are able to eliminate the parasite before the infection becomes clinical though the complete mortality after week 16 implies that these oysters may be succumbing to infection before the disease has left its latent stage, possible as a result of an immune system overreaction. Loch Ryan stock may be a resistant stock or a severely susceptible stock.

It is clear that after over 40 years of studying the protozoan parasite, the full life cycle of *Bonamia ostreae* or the complete means by which it may spread is still unknown. Though several studies have concluded that other species of macroinvertebrates do not act as carriers for *B. ostreae* (van Banning 1987, Figueras & Robledo 1994, Renault et al. 1995, Culloty et al. 1999), the recent advancements in molecular techniques have led to the detection of the disease in species such as *Ophiothrix fragilis, Crassostrea gigas* (Lynch et al. 2007, 2010) and in this study, *Mytilis edulis* (Chapter 4). In Chapter 4, larvae of *Ostrea edulis* were also discovered to carry the disease. As larvae are motile, this may also be a means of disease spread. Perhaps with continued screening using modern molecular techniques additional species will be found to carry the disease. Though, in future, other species may be found to carry the parasite, further research into these animals is essential to determine if the do, in fact, spread the disease or simple carry it within their tissues. As further discoveries continue to be made in terms of the life cycle and

transmission, caution is critical in the movement of all animals and equipment from *B. ostreae* infected areas.

Chapter 2 again highlights the fact, found by several studies (da Silva & Villalba 2004, Lynch et al. 2008), that the methods by which the disease is diagnosed, recommended by the OIE (OIE 2010), may not be 100% reliable. This leads to great difficulty if the continual spread of the bonamiosis is to be prevented. It appears that it may be necessary for standardised protocols and methodologies to be introduced by the OIE or EU for *B. ostreae* diagnosis, especially with regards to molecular methods, which can vary greatly from laboratory to laboratory. The discovery, in Chapter 5**,** of *B. ostreae* infection, by ISH, in oyster samples determined negative by the commonly used diagnostic methods of heart imprints and PCR analysis, has highlighted the issue of disease spread by new, latent infections, again the possible failings of current diagnostic methods and the need for a better, combinative screening method.

In terms of conservation, the studies here indicate that eradication of the disease is not a feasible option, the focus must be moved to the proper management of currently existing beds rather than the breeding of a single resistant stock or the movement of successful stocks to depopulated areas, as it is clear from previous studies, such as Culloty et al. (2004) and the work in Chapter 3**,** that oyster populations have strong adaptations to their environments and that, with some husbandry and stock management, they may be able to recover their numbers somewhat, even in the face of *B. ostreae* infection.

In conclusion, it appears that the conservation and recovery of *O. edulis* is possible. To move forward with conservation, the development of localised hatcheries and proper husbandry management of *O. edulis* populations, along with more cautious

guidelines in terms of diagnostic methods and movement of shellfish and equipment within an *O. edulis* area is required.

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