Synecology in soft sediment bivalves: the influence of parasites, physiological processes, and environmental stressors on health and disease.

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DECLARATION

The thesis submitted is my own work, it has not been submitted for another degree, to any other universities.

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Table of Contents

ABSTRACT
Ecosystem goods and services provided by estuarine and near coastal regions are being increasingly recognised for their immense value, as is the biodiversity in these areas. Dominant bivalves in intertidal regions, such as *Cerastoderma edule* (common cockle) and *Mytilus edulis* (blue mussel), not only are commercially exploited, but also are ecologically important, supporting a range of other species as a food source, as a link between trophic levels and as a habitat for a range of parasites. The marine environments are altering rapidly due to climate change, and near coastal communities have been identified as sentinels of change. Population structure and reproductive biology of two bivalve molluscs the cockle, *Cerastoderma edule* and the blue mussel, *Mytilus edulis* were assessed at two study sites over a 16-month study period to consider on-going alterations in the thermal regime. Two sites 215 km apart were chosen, Flaxfort Strand, Co. Cork and Bannow Bay, Co. Wexford. Both sites are classified as Special Areas of Conservation and Special Protected Areas under the EU Birds and Habitats Directives. Following an anomalously harsh winter – the coldest in 50 years in Ireland, in the midst of several mild years, advancement of spawning time was observed in both species. Furthermore the cockles underwent gametogenesis over winter in Ireland. This contrasts with previous studies here, and spawning was of greater duration than other historical studies, which may indicate modifications in reproductive biology in response to climate change. Throughout Ireland and Europe the cockle has experienced mass surfacings and mortalities in geographically distinct regions, with various causes proposed. Mass surfacings have been observed in Ireland previously and a 16-month study of cockles again at the two same aforementioned sites was undertaken to explore this phenomenon. Surfaced (moribund) and buried cockles were collected on a monthly basis from Flaxfort Strand and Bannow Bay and their health compared using histology and cPCR. A range of parasites and one pathological condition was identified. Age was highlighted as a source of variation between dying and healthy animals with a parasite threshold being reached possibly around age three. Prevalence of disseminated neoplasia and trematodes, in combination, differed significantly between surfaced and buried cockles at both sites. However local factors dominated when looking at the cause of surfacing at each site, as it was specific to each location as the prevalence and intensity of parasites

varied at both. The health of mussels was explored too on a temporal and seasonal basis in an attempt to assess what constitutes a healthy organism or are parasitised animals still healthy. Despite being parasitized mussels continued to reproduce and did not experience significant mortalities. In essence external drivers can tip the balance between "acceptable" levels of infection where the mussel can still function physiologically and "unacceptable" where prevalence and intensity of infection can result in physiological impairment at the individual and population level. Synecological studies of intertidal ecosystems are lacking, so all other bivalves encountered during the sampling were assessed in terms of population structure, reproduction, and health (histology and PCR). *Macoma balthica*, *Angulus tenuis*, *Scrobicularia plana*, *Mya arenaria*, and a single *Ruditapes* specimen were collected from the sediment at both sites. From this, and the aforementioned studies of cockle and mussel health performed, the range of parasites in the intertidal area was shown. Trematodes and ciliates dominated in terms of species. It became clear too, that some parasites might specialize on one host species (e.g. *Meiogymnophallus minutus* metacercariae and cockles) while others (e.g. *Nematopsis* sp.) are not so specific in host choice. Furthermore the population genetics of the cockle, it's most common parasite on Irish shores *Meiogymnophallus minutus*, and its hyperparasite *Unikaryon legeri* were examined too. Low levels of genetic differentiation have been observed in many studies of cockles throughout Europe, therefore this study investigated whether populations could be differentiated on a genetic basis by their parasites or hyperparasites. Genetic studies of digenean trematodes are rare, and the hyperparasite is known from morphological work only. Samples from Ireland, Wales, France, and Morocco were collected and analysed. Different extraction techniques were used and this may have been shown in the quality of DNA rendered, however a small nucleotide polymorphism was detected upon comparison of Ireland and Morocco.

CHAPTER 1: GENERAL INTRODUCTION

1.1 *Estuarine and near coastal communities*

Estuaries exist at the meeting of freshwater rivers, land, and the sea; due to the meeting of these three ecosystem types the value of estuaries is greater than the sum of its parts (James *et al*., 2007; Barbier *et al*., 2011). Ecosystem value attributed to estuaries and near coastal regions can be divided into three types (i.) goods e.g. water, and other products from the ecosystem, (ii.) services e.g. water purification, recreation and climate regulation, (iii.) cultural benefits e.g. religious beliefs, heritage values (Barbier *et al*., 2011). The value of coastal marine ecosystems has been placed at US $ 14 trillion per year (Harley *et al*., 2006). Different estimates in different places demonstrate how the world's settlements are concentrated in coastal areas with 40 – 60 % of the world's population living within 100 km of the coast (Vitousek *et al*., 1997; Hauxhall *et al*., 2003). This value will increase nonetheless as growth of coastal populations is greater than in inland areas (Hauxhall *et al*., 2003).

Non-human inhabitants of these areas may be either ephemeral, using them as part of their development, e.g. as nursery areas for commercially fished species such as whiting (*Melangius merlangus*) and cod (*Gadus morhua*) or constants such as shellfish (Wilson, 2002). It is estimated that 37 % of commercial fish in Europe use estuaries at some stage of their life cycle (Wilson, 2002). Additionally commercially exploited shellfish such as cockles *Cerastoderma edule* and mussels *Mytilus edulis* reside in estuarine and near coastal areas.

1.2 *Role of bivalves in soft sediments*

Ecosystem engineers are defined as organisms that alter the physical environment with concurrent effects on other species (Reise, 2002). Bivalve molluscs such as *Cerastoderma edule* and *Mytilus edulis* may act as both autogenic and allogenic ecosystem engineers – as their shells and biogenic structures provide habitat and by their feeding mechanism as suspension feeders they recycle nutrients and deposit organic matter (Coleman and Williams, 2002; Reise, 2002). As suspension feeders, they too are responsible for a large portion of the energy transport and biomass in an ecosystem (Coleman and Williams, 2002). Bivalves are integral in the cycling of nutrients between the sediment and the water column (Whiteley and Bendell-Young, 2007). They exploit primary producers (plankton) and as such they are a linkage to higher trophic levels as

crabs and fish too predate upon them (benthic coupling) (Whiteley and Bendell-Young, 2007).

1.3 *Parasites in estuarine/near coastal areas*

Estuaries can be described as hotspots of biodiversity; however a large portion of this diversity may be ignored, as it is in the form of parasites. All free-living organisms can harbour parasites, and parasitism is the most commonly adopted life style on earth equating to 40 % of all recognised species (de Montaudouin and Lanceleur, 2011; Nichols and Gomez, 2011; de Montaudouin *et al*., 2012). Despite their hidden discrete nature, the intertidal region is dominated by digenean trematode parasites and their presence can shape communities by their influence over hosts (Goater, 1993; Thieltges *et al*., 2006: Lassalle *et al*., 2007; Dubois *et al*., 2009; Studer and Poulin, 2012). There are a multitude of effects as a result of parasitism, not least enhanced mortality in the case of digenean trematode sporocysts, but also non-lethal results such as reduced growth, reduced fecundity and increased sensitivity to anoxia (Wegeberg and Jensen, 1999; Secor *et al*., 2001). However many studies of ecosystems do not take parasites into account, even though parasites impact upon hosts and their populations (Kutz *et al*., 2009).

1.4 *Current knowledge on climate change*

The Intergovernmental Panel on Climate Change (IPCC) has defined climate change as "a change in the state of the climate that can be identified by changes in the mean and/or variability of its properties, and that persists for an extended period, typically decades or longer" (Kutz *et al*., 2009). By this definition few could deny that climate has altered over the course of the 20th and into the 21st century and this modification is expressed in a range of ways including temperature, precipitation, and extreme events (Kordas *et al*., 2011). This is a global phenomenon and at a broad scale it impacts upon commercial enterprises, such as fisheries, human settlements, and all species inhabiting or exploiting the marine environment. Indeed alterations in fisheries productivity have already been felt, with decreases in yields in the Celtic Biscay Sea area but an increase further north in the Norwegian Seas (Philippart *et al*., 2011).

Sea level rise attributed to climate change is also evident, as a result of thermal expansion and ice sheet/glacier melting (Hoegh-Guldberg and Bruno, 2010). It is predicted that sea levels around Ireland will increase by 18 – 59 cm in the 21st century. The IPCC reports that from observational data, there has been an increase in the North Atlantic of tropical cyclone activity since circa 1970. This, in combination with sea level rise may lead to a reduction of water quality, as they could facilitate re-entry of sediment bound contaminants (Schiedek *et al*., 2007).

1.5 *Estuarine areas and climate change*

The implications of such rapid and indeed extreme alterations in climate undoubtedly have consequences for all that inhabit and exploit the marine environment. Recent studies have highlighted estuarine and coastal communities as indicators of climate change (Madeira *et al*., 2012). From 1961 globally ocean temperatures have increased by 0.10 °C from the surface to a depth of 700 m (IPCC). The maximum increase predicted by the year 2100 is 4°C (Callaway *et al*., 2012). Around Ireland the seas have been warming by 0.3 to 0.4 C per decade since the 1980s, however this increases to 0.6 – 0.7 C in the Irish Sea (Dunne *et al*., 2013). However these lowland areas may also be impacted by climate induced sea level rise, for instance European seas border 68, 000 km of coastline and worldwide estuaries and near coastal areas provide ecosystem goods and services totalling $14 trillion US dollars per annum (Harley *et al*., 2006; Philippart *et al*., 2011). In northern latitudes modifications in habitat are being caused primarily by climate change (Kutz *et al*., 2009). Therefore it is essential to focus upon estuarine areas and their communities as indicators of climate change but also gain a greater understanding of their functioning to attempt to mitigate against the ill effects of climate variability.

One of the most conspicuous effects of climate change is alteration in the thermal regime. Temperature, especially in the case of ectotherms is a key aspect in determining range; the tolerance window of an organism in terms of temperature is a "favourable zone" (Madeira *et al*., 2012). Continuing increases in temperature and the swift nature of the changes that have already occurred and that are on going may mean that many species are already out of their favourable zone (Schiedek *et al*., 2007). This will lead to changes in the community

structure in a given area. Changes in physiology, behaviour, growth, reproduction, and recruitment may be caused by climate change (Fujii, 2012) and 15 year declines in some intertidal bivalve species in the Dutch Wadden Sea (*C. edule*, *Mytilus edulis*, *Mya arenaria*, and *Macoma balthica*) have been attributed to a series of mild winters (Honkoop *et al*., 1998; Philippart *et al*., 2003; Beukema and Dekker, 2005). As the declines in species are in both commercially exploited and non-commercial bivalves, the effects will be felt economically and ecologically. Changes in species range may be evident too. The range of the Baltic tellin, *Macoma balthica* is believed to have moved 300 km northwards over the preceding 35 years – it is no longer found in the southern part of the Bay of Biscay which was the limit of its southern distribution previously (Jansen *et al*., 2007).

According to Bibby *et al*. (2008) in the region of half of the CO_2 produced as a result of human activities has been absorbed by the world's oceans. Consequently acidification of the world's oceans is occurring, and a decrease in pH of 0.1 units has been recorded since pre industrial levels (Bibby *et al*., 2008). The increased concentration of hydrogen ions and decreases carbonate ions in seawater. Reductions in carbonate ions will have concurrent effects for organisms such as bivalves that require carbonate for shell building. Additionally reproduction and immune parameters may be impacted negatively too (Bibby *et al*., 2008). Seawater with a pH lower than 7.5 permanently lowered haemolymph pH, growth and metabolism. Similarly gonads may be resorbed to reduce energy expenditure under acidic conditions or spawning may be delayed (Bibby *et al*., 2008).

Climate associated phenological changes have been observed in taxa e.g. spawning in *Macoma balthica* in the Dutch Wadden Sea has advanced one day per year in the last 20 years (Visser and Both, 2005). These phenological changes are complex as is their interpretation – on one hand they may reflect adaptation to climate, but they also show that climate is altering the timings of seasonal events e.g. breeding (Visser and Both, 2005). Climate variation is not a new phenomenon and therefore phenotypic plasticity is observed in life history (Visser and Both, 2005). Phenotypic plasticity is one of the ways species may react as their environment changes due to climate (Silvestre *et al*., 2012). The others include extinction/extirpation, adapt as a consequence of alterations in

genetic composition or migrations (Silvestre *et al*., 2012). Phenotypic plasticity may be more difficult in animals with longer generation times (Silvestre *et al*., 2012), but in bivalves this may be a viable option.

1.6 *Climate change and disease development*

Parasites and pathogens in terrestrial and marine systems are influenced by climate, and their diversity and abundance may be modified by climate change (Kutz *et al*., 2009). In the terrestrial realm, a warmer climate, with increased precipitation, may lead to increased transmission rate, longer transmission window of opportunities and changes in disease range and timing of outbreaks (Kutz *et al*., 2009).

As a consequence of climate change the virulence, range, growth rates, and effects of some pathogens such as marine fungi and bacteria (e.g. vibrios) may be enhanced (Harvell *et al*., 2002). It is thought that the prevalence of disease in the marine environment is increasing over the last three decades, but there is a lack of baseline data for comparative purposes (Ward and Lafferty, 2004). Greater prevalence of disease may be linked to thermal stress, increased pollution due to climate change, range expansions of pathogens, and their vectors (Ward and Lafferty, 2004; Hoegh-Guldberg and Bruno, 2010).

Additionally the impact of, transmission, and range of parasites may be altered in turn by climate change (de Montaudouin *et al*., 2009). The transmission of parasites especially in temperate regions is performed during a temperature specific time period, warmer winters could allow for greater transmission time (Hakalahti *et al*., 2006). The diversity of parasites may change too, as range expansions and local extinctions may take place (de Montaudouin *et al*., 2009). Furthermore for parasites with complex life cycles who depend on several hosts, one or more of the intermediate hosts may decrease in number and completion of their life cycle may be impeded (Rohr *et al*., 2011). Currently the range of parasites (both micro and macro) in many soft sediment bivalves in Ireland is unknown, thus it is impossible to estimate alterations in parasite diversity due to climate change, or if this is occurring or to estimate their effects on the host and subsequent community.

1.7 *SUSFISH Shellfish productivity in the Irish Sea: Working towards a sustainable future*.

This PhD work was undertaken as part of the wider SUSFISH project. This is a collaborative multidisciplinary project, which is bringing together experts in economics, genetics, immunology, physiology and health from the University's of Bangor, Aberystwyth and Swansea in Wales and University College Cork. The aim of the project is to investigate the effect climate change will have on the Irish Sea in the next 50-100 years and to provide guidelines for management and policy.

1.8 AIMS AND OBJECTIVES

- Examine the reproductive biology of the commercially and ecologically important species *Cerastoderma edule* and *Mytilus edulis* (Chapter 3 and 4).
- To compare these findings against previous studies to gauge if recent climate variability has resulted in alterations in reproductive strategy (Chapters 3 and 4).
- Investigate the population structure and health status of other soft sediment bivalves, which by their non-commercial nature may not be normally included in studies despite contributing to the ecosystem as a whole (Chapter 4). To investigate mortality events in the cockle with particular emphasis on endogenous and exogenous factors that might be driving development of disease and subsequent mortalities (Chapter 5).
- To conduct a comprehensive health screen of *Mytilus edulis,* using histology and molecular means since such studies are lacking in Ireland and abroad, despite its commercial exploitation (Chapter 6).
- Create an atlas of parasites and pathological conditions in common soft sediment bivalves and to investigate biodiversity and parasite burden in these bivalves (Chapter 6).
- To attempt to further our knowledge by molecular techniques of *Cerastoderma edule*, its most common parasite on Irish shores *Meiogymnophallus minutus* and the hyperparasite *Unikaryon legeri*. In the case of *M. minutus* and *U. legeri* molecular data are lacking, despite the importance of parasites as selective forces and their undoubted influence on the host (Chapter 7).

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CHAPTER 2: MATERIALS AND METHODS.

2.1 Study sites

2.1.1 *Flaxfort Strand*

Two sites 215km apart were selected. Flaxfort Strand (N51°38' 47.4", W 008°41'45.0") is a small semi-exposed bay on the south west coast of Ireland (Figure 2). It is composed primarily of sand flats and extensive colonisation by green algae of *Enteromorpha* spp. can be seen on the upper shore during the summer months. The tidal range is 4.2 m. The River Argideen drains into the area and the water quality is classed as eutrophic by the Environmental Protection Agency (www1). Land usage of the surrounding areas is predominantly dairy farming. Flaxfort Strand is part of Courtmacsherry Bay, which is designated as a Special Area of Conservation (SAC: 001230). It was allocated this status as ten habitats recorded on Annex 1 of the EU Habitats Directive (www2) make up this bay. Clonakilty Bay, which is adjacent, is classed as a Special Protected Area (SPA: 004081); it includes habitats classed as Annex 1 on the EU Habitats Directive (www3) plus bird species with Annex 1 status on the EU Birds Directive e.g. bar tailed godwits and golden plovers frequent this area.

2.1.2 *Bannow Bay*

Bannow Bay (N52°13'07.9", W006°46'37.8") is located on the south east coast of Ireland; it is a shallow bay that is over ten times larger than Flaxfort Strand. It has an area of ~1050 hectares and at its greatest is 8km in length and varies between 1 and 3 km wide and had a tidal range of 3.6 m. Water stays in the bay for two tidal cycles and the bay itself is comprised of a mixture of gravel, sand and mudflats (Cotter *et al*., 2010). Unlike Flaxfort Strand, large banks of *Entermorpha* spp. were not observed for the duration of the study and according to the National Parks and Wildlife Service its habitats are of "general good quality" (www4). It is classified as a Special Protected Area – Site Code IE 0004033 under the EU Birds Directive as it too provides habitat for several Annex 1 bird species (the golden plover, bar tailed godwit) and there are two breeding Annex 1 species present, the little tern, and the kingfisher. Under the EU Habitats Directive (92/43/EEC) it is classed as a Special Area of Conservation (Site Code IE000697) as it encompasses 11 Annex 1 habitats as decreed by the Habitats Directive (www4). Furthermore it is also named as a National Heritage Area (NHA Site Code 0000697) and subject to the European Communities (Quality of Shellfish Waters) Regulations S.I No. 268 of 2006 European Communities (Quality of Shellfish Waters) Regulations 2006.

Table 1: Site characteristics of Bannow Bay and Flaxfort Strand.

Characteristic	Bannow Bay	Flaxfort Strand
Size	8 km length, 3 km width	1 km length, 0.5 km width
Exposure Level	Enclosed	Semi-exposed
Retention	High	
Tidal Range	3.6 m	4.2 m
Tidal Exchange	Slow	Rapid
Water Quality	Good	Poor
Composition	Mud flats and sand	Mud flats, coarse and fine sand
Macroalgal Growth	Minimal	Extensive
Surrounding Land Usage	Farmland, settlements, secondary roads	Farmland, settlements, primary roads

2.2 *Temperature loggers*

One Stowaway ®Tidbit ™ Weatherproof and Waterproof Temperature Logger was placed in a channel of water at each site, adjacent to the main collection area. As the loggers were in a tidal channel they were emersed and immersed with the bivalves under study. The temperature was recorded every 16 minutes from their deployment until the cessation of the study. The data were retrieved using an Optic Base Station/Coupler Kit and analysed with BoxCar®Pro 4.3 Starter Kit software. Additional temperature data for the study period were provided for Bannow Bay by Met Éireann from Johnstown Castle (20.9 km away) and for Flaxfort Strand by the Marine Institute from Bantry Bay (distance of 65.5 km) and Met Éireann from Cork airport (40.5 km from Flaxfort Strand) (Figure 1).

Figure 1: Map of Ireland plus study locations Bannow Bay and Flaxfort Strand with additional temperature stations included (aerial photos taken at 500 m). 1. Johnstown Castle, 2. Bannow Bay, 3. Cork Airport, 4. Flaxfort Strand, 5. Bantry Bay.

2.3 *Biology*

2.3.1 *Cerastoderma edule*

Cerastoderma edule, the common cockle is a widely distributed infaunal, bivalve mollusc from Mauritania in West Africa to as far north as Scandinavia. Throughout its range it can be locally very abundant, it withstands a wide range of tidal strengths and salinities but is sensitive to low oxygen and extremes of temperature (Reise, 2003).

It is commercially fished in Ireland and abroad in the UK and France and cockle shells have been found in Viking and earlier middens (www5; Wilson, 2002). The largest yields were previously landed in the Netherlands, however there have been decreases due to curtails in the fishery with the banning of mechanical fishing in the Wadden Sea since January 2005, and further restrictions on sand flats due to a report on the impacts on sediment and other bivalves (Ens *et al*., 2004; Clarke and Tully, 2011). The total tonnage landed has decreased from a peak of 106, 494 tonnes to 9, 316 tonnes in 2010 in Europe (the last year there are statistics available) (www5).

Recruitment in cockles is known to vary markedly in success between years and sites, with large increases in density occurring over a short period of time (Ramon, 1996; Honkoop *et al*., 1998; Sauriau and Kang, 2000). As aforementioned declines in cockle recruitment have been attributed to milder winters – following colder winters greater recruitment has been recorded which is attributed to a predator prey mismatch between cockle juveniles and their predators *Carcinus maenas* and *Crangon crangon*. The last study of *C*. *edule* reproduction occurred in Strangford Lough in Northern Ireland in the 1970s, and showed rapid gametogenesis in spring followed by spawning in spring/summer (Seed and Brown, 1977).

Up to 16 species of digenean trematode, plus copepods, turbellaria, gregarines, and a range of bacteria inhabit *Cerastoderma edule* over the course of its range (Carballal *et al*., 2001; de Montaudouin *et al*., 2009). Previous research in Ireland using whole body squashes (Fermer, 2010) has concentrated on the digenean trematodes found in *Cerastoderma edule*, and identified eight species of trematode in Irish cockles. *Meiogymnophallus minutus* (synonym *Parvatrema minutus*) was the most common trematode in terms of prevalence and intensity in southwest Ireland. The concept of the cockle as a microhabitat has been proposed (de Montaudouin *et al*., 2009) and is quite apt as different trematode species are confined to specific tissues e.g. *M*. *minutus* in the hinge region, *Gymnophallus choledochus* and *Bucephalus minimus* in the gonads, and *Renicola roscovita* in the palps (de Montaudouin *et al*., 2009). *Himasthla elongata* and *H*. *interrupta* colonise the foot, and in the case of *H*. *interrupta* the mantle too, while *Psilostomum brevicolle* is restricted to the visceral mass (de Montaudouin *et al*., 2009).

One pathological condition, disseminated neoplasia, is common in cockles from some regions and it was first identified in cockles from Cork Harbour in 1982 and later that year in Brittany, France (Twomey and Mulcahy, 1988; Villalba *et al*., 2001; Romalde

et al., 2007). Disseminated neoplasia is of unknown origins – but a viral aetiolgy has been hypothesized (Romalde *et al*., 2007). It is characterized by the proliferation of highly invasive, mitotically active, large pleomorphic haemoyctes (NOAA Technical Memorandum NMFS-NE-107, 1992; Krishnakumar *et al*., 1999; Collins and Mulcahy, 2003; Barber, 2004; Ciocan *et al*., 2006; LeGrand *et al*., 2010). The areas from which neoplastic cells arise have yet to be conclusively determined (Carballal *et al*., 2001). It can be a lethal condition and mortality has been observed within 3-5 months, remission is possible but only in the initial stages of the disease (Twomey and Mulcahy, 1988; Collins and Mulcahy, 2003; Ciocan *et al*., 2006; Romalde *et al*., 2007; LeGrand *et al*., 2010).

Mass surfacing and mass mortalities in cockles have been observed in cockles in geographically distinct regions. Surfacing in cockles has been described as a "prelude to death" and is an indicator of ill health (Blanchet *et al*., 2003). Perhaps one of the most well-known cases of cockle losses is in the Burry Inlet, Wales due to the large economic costs that ensued. Haplosporidia in addition to trematodes and neoplasia are believed to have contributed to these mortalities (Elliot *et al*., 2012). Trematodes and neoplasia have been implicated in previous cockle mortalities in Galicia Spain in 1997 (Carballal *et al*., 2001) and Cork, Ireland in 2009 (Morgan *et al*., 2012). Trematodes may have been responsible for mortalities in the northern Wadden Sea in 2004 (Thieltges, 2006). Although some common causes of mass mortalities are evident (e.g. trematodes) there does appear to be variation among sites and this warrants further investigation – especially considering the key role cockles play in the ecosystem as habitat, food source, and a link between trophic levels (Morgan *et al*., 2013).

2.3.2 *Mytilus edulis*

Mytilus edulis is holarctic in distribution (Secor *et al*., 2001) and lives on all European coasts with hard substrates (www 6). It can be found in a range of habitats from the intertidal to a depth of 40 m including rocky shores, estuaries, and man made structures such as piers (www6). As evident from its wide distribution *M. edulis* can withstand a wide range of salinities, temperatures, and variation in oxygen levels (www7). It plays a part in nutrient cycling and other biochemical processes and mussel beds/production areas may support a wide range of bird species (Roycroft *et al*., 2004).

Capture fisheries and aquaculture exploit *Mytilus edulis*. Peak yields in the capture fishery lead to the landing of 170, 580 tonnes in 1993, which has declined to 50, 148 tonnes in 2010 (www 6); high yields are continuing via aquaculture and 207, 918 tonnes were produced in 2010 globally (www6). It is the most common bivalve produced by Irish aquaculture and the second most common species produced through aquaculture overall in Ireland (Roycroft *et al*., 2004). Mussel aquaculture relies upon wild seed (www3; Domínguez *et al*., 2010) but hatchery techniques are available for its production via mussel conditioning (www7). Various ongrowing techniques exist for the culture of mussels these include: onbottom culture, Bouchot culture, raft, and the most recent development longline or rope culture (www7).

Preceding studies of mussel pathology in Ireland have focused their efforts on the trematode *Echinostephilla patellae* in the mussel (Prinz *et al*., 2010). However a range of other parasites have been identified in mussels across their range which may impede production these include the copepods *Mytilicola intestinalis*, *M. orientalis*, microsporidia e.g *Steinhausia mytilovum* and various viruses e.g. Picornaviridae and bacteria such as *Chlamydia*-like bacteria and *Rickettsia*-like bacteria (www7; www8). Even though an assortment of parasites and disseminated neoplasia have been documented in mussels, the reports of mass mortalities that occur for cockles do not appear to do so for this species. Although there have been studies of mussel pathology previously (e.g. Bignell *et al*., 2008) but there is a paucity of knowledge concerning mussel pathology and furthermore studying the health status of commercially important bivalves is important for management of wild and farmed stocks (da Silva *et al*., 2002; Boehs *et al*., 2010).

2.3.3 *Macoma balthica*

Macoma balthica, the Baltic tellin, is a common bivalve distributed on both sides of the Atlantic, from Norway to Arcachon Bay, France, and from Greenland to South Carolina in the US (Beukema and Meehan, 1985; Hummel *et al*., 1996; Kamermans *et al*., 1999; Jansen *et al*., 2007; Long *et al*., 2008). It can be present in high densities and in Chesapeake Bay, 85 % of the total biomass in muddy mesohaline areas is composed of *M. balthica* (Long *et al*., 2008). It is subject to heavy size dependent predation – larger tellins (>20 mm shell length) are predated upon by birds e.g. oystercatchers and those that are less than 15 mm are targeted by crabs (Griffith and Richardson, 2006). In Chesapeake Bay, *M. balthica* makes up 50 % of the diet of the blue crab *Callinectes sapidus* (Long *et al*., 2008). As predator avoidance it can bury

deeper in the sediment but this alters its feeding capacity, and thus siphon size and growth rate (Griffith and Richardson, 2006). It has long feeding siphons and it can alternate between suspension and deposit feeding (Griffiths and Richardson, 2006). The number of spawning events depends on latitude. In northern locations they spawn in spring. Further south in France, spring spawning is followed by redevelopment, and further spawning in September/November (Günther *et al*., 1998). It is the first and second intermediate host for the trematode *Parvatrema affinis*, whose final host is gull and other birds (Pekkarinen, 1984), and it is also parasitized by *Gymnophallus gibberosus*, *Lacunovermis macomae* and the echinostomatid, *Echinostomatia cercaria* (synonym Himasthla elongata) (Pekkarinen, 1984).

2.3.4 *Angulus tenuis*

Angulus tenuis (= *Tellina tenuis*) the thin tellin is a suspension feeder found in the intertidal to the sublittoral (www9; Trevallion, 1971). It buries from 50 – 120 mm deep, its long siphon is used to feed, and this can be removed by feeding flatfish e.g plaice (www9; Trevallion, 1971) (but it has the capacity to regenerate). In the intertidal *A*. *tenuis* is found south of 56 °N along Atlantic coasts and the continental North Sea coast (Dekker and Beukema, 1999). It has a more widespread distribution in subtidal regions, from Northern Norway to the Atlantic coast of Morocco and into the Mediterranean and Black Sea areas (Dekker and Beukema, 1999). It can reach high densities in sandy, sheltered beaches and has a long life span of eight years (Dekker and Beukema, 1999). In parts of France sporocysts and cercariae of *Meiogymnophallus striagus* have been identified in *A*. *tenuis* (Bartoli, 1983).

2.3.5 *Scrobicularia plana*

Scrobicularia plana, the peppery furrow shell, is a widespread estuarine bivalve; it has a distribution encompassing Norway to Senegal, and into the Mediterranean excluding the Black Sea (Rodriguez-Rua *et al*., 2003). It is a dioecious, deposit, and suspension feeder that prefers muddy sediments with high organic matter content (Rodriguez-Rua *et al*., 2003; Chesman and Langton, 2006; Santos *et al*., 2011). It buries to 200 mm, and it is an important part of the estuarine food web (Sola, 1997). Sexual maturity is reached in the second year or at shell length 20 mm (Rodriguez-Rua *et al*., 2003). Developing and spawning gonads have been observed from spring to late autumn, and gonads remain inactive from October to January in Spain (Rodriguez-Rua *et al*., 2003) however latitudinal variation in spawning is observed (Sola, 1997). It is the first intermediate host of the digenean trematode

Meiogymnophallus minutus that then utilizes *Cerastoderma edule* as a second host, and the oystercatcher as its final host (Russell-Pinto *et al*., 1996).

2.3.6 *Meiogymnophallus minutus*

Meiogymnophallus minutus is the dominant trematode species in cockles, *Cerastoderma edule*, on Irish shores (Fermer, 2010). Its first intermediate host is the peppery furrow shell *Scrobicularia plana* in which the cercarial stage develops (Russell-Pinto, 1990). Cercariae can then colonise the second intermediate host the cockle via several routes: the inhalant siphon, the gills, or the haemolymph (Russell-Pinto *et al*., 1996). Cercariae are then encapsulated by the mantle epithelium, after they move to the wedge shaped cavity in the hinge (Russell-Pinto, 1990; Russell-Pinto *et al*., 1996). The final hosts are the oystercatcher *Haematopus ostralegus* or *Melanita nigra*, both of whom consume cockles (Russell-Pinto, 1990).

2.3.7 *Unikaryon legeri*

The microsporidian *Unikaryon legeri* has been identified as a hyperparasite of the trematode *Meiogymnophallus minutus* (Azevedo and Canning, 1987; Fermer *et al*., 2009). They infect the metacercariae and appear to be fatal; the microsporidian spores make the metacercariae enlarge to in some cases twice the normal size and alter their coloration to opaque/greyish white (Bowers and James, 1967).

2.4 *Sampling*

The same methodology was employed at both sites and fieldwork was carried out at monthly intervals commencing in March 2010 in Bannow Bay and April 2010 in Flaxfort Strand, continuing until June 2011 at both sites. Due to adverse weather conditions samples were not collected from Bannow Bay in December 2010.

2.4.1 *Sampling of cockles*

30 buried cockles from within the sediment, and 30 surfaced moribund cockles (gaping and incapable of closing) were collected monthly from each site at low tide. Cockles in standing water were not sampled for this study; only those that remained buried at low tide were included in the study of reproduction – all cockles were considered in the study of cockle pathology.

2.4.2 *Sampling of mussels*

Each month 24 - 30 mussels were collected from rocks and large stones embedded (10 – 20 mm) in the muddy sediment at both sites. Mussels from Flaxfort Strand were collected from the mid shore, while those from Bannow Bay were found on the

upper shore, as these were the areas at each site where mussels were locally most abundant. Often the mussels were covered by seaweed such as fucoids and also barnacle species. The overall collection area at both sites; varied by up to 10 metres horizontally upon each sampling occasion.

2.4.3 *Sampling of other bivalves*

As buried cockles were excavated any other bivalves encountered were taken too. *Macoma balthica*, *Angulus tenuis*, *Scrobicularia plana*, *Mya arenaria* and a *Ruditapes* specimen were collected.

2.5 *Morphometrics and age structure*

The whole weight, shell weight, and tissue weight were weighed to two decimal places (g). The number of growth rings deposited on the cockle shell upon cessation of growth each winter were counted (Hancock, 1967).

2.6 *Condition Index*

The mean condition index was calculated on a monthly basis. The average wet tissue weight and average wet shell weight were used as per an altered version of Drummond *et al*. (2006) and Cotter *et al*. (2010) (as they used dry weights).

$$\frac{Condition\ Index\ =\ Wet\ Tissue\ Weight\ (g)\ x\ 100}{Shell\ Weight\ (g)}$$

2.7 *Histology*

In the laboratory, bivalves were opened carefully and a transverse section through the gonad regions were preserved for histology in Davidson's Solution (Shaw and Battle, 1957), and refrigerated for two days at 4˚C. They were sectioned at 5µm and stained with Haematoxylin and Eosin. The slides were examined at 4x, 10x, and 40x magnification.

2.8 *Reproduction*

2.8.1 *Cerastoderma edule*

Sex was determined if gonads were present and gonadal maturity stages were assigned to each of the cockles. Five reproductive stages were identified based on a

scale from Drummond *et al*. (2006), which was modified to reflect differences between clam and cockle morphology. These were: early developing, late developing, ripe, spawning/partially spent and spent (Figure 3, tables 1a and 1b). When cockles presented stages that could be described as intermediate between two stages, the stage that dominated the greatest part of the gonads was assigned.

2.8.2 *Mytilus edulis*

The sex and gonadal stage (Table 3a and 3b) were noted, based on scales modified from Seed (1969), Drummond *et al*. (2006) and Morgan *et al*. (2013). Five stages of gonadal maturity were identified.

2.8.3 *Macoma balthica*

Three stages of gonadal maturity were assigned to *Macoma balthica* that had developed gonads. These were developing, ripe/spawning and spent. These were based on scales from Drummond *et al*., (2006) and Morgan *et al*., (2013).

2.8.4 *Angulus tenuis*

Four reproductive stages were recognised in *Angulus tenuis*, these were modified gonadal stages from Drummond *et al*., (2006) and Morgan *et al*., (2013).

Table 2a: Description of female gonads based on a modified scale from Drummond et al. (2006).

Stage	Description
Early Developing	Small number of oocytes in discrete areas visible with none free in the lumen. Dark in colour and with grainy borders.
Late Developing	Larger area taken over by oocytes which have increased in size – still some small in size, with a more defined shape. Some now free from the follicle wall, but remaining in discrete pattern.
Ripe	Majority of the section containing oocytes, which are now more uniformly large. Oval in shape and free in the lumen with thin barriers between oocytes. Appear in distinct net pattern.
Spawning/Partially Spent	Broken follicle walls, the number of oocytes reduced. Spaces beginning to show in the gonad region, some oocytes remain that are no longer in a homogenous pattern.
Spent	Section through gonads appears broken, with large spaces evident. Remaining oocytes dark in colour and isolated, with no net-like pattern remaining.

Table 2b: Description of male gonads based on a modified scale from Drummond et al. (2006).

Stage	Description
Early developing	Gonads have begun to develop, small amount discernible in plugs. Comprised mainly of lighter, rounder and larger spermatogonia encompassing smaller darker spermatocytes. Still appear quite loose in composition.
Late developing	Still mainly spermatogonia and spermatocytes, but some elongating spermatids visible in the centre of the plug. Spermatids stained navy blue in comparison to purple spermatogonia and spermatocytes.
Ripe	Plugs of gonad very organised and intact more compacted than earlier stages. Mainly spermatozoa, tails of the spermatozoa facing the lumen.
Spawning/Partially spent	Spermatozoa leaving plugs, empty spaces visible. The neat organised structure degrading.
Spent	Small amounts of sperm visible scattered through section, follicles broken.

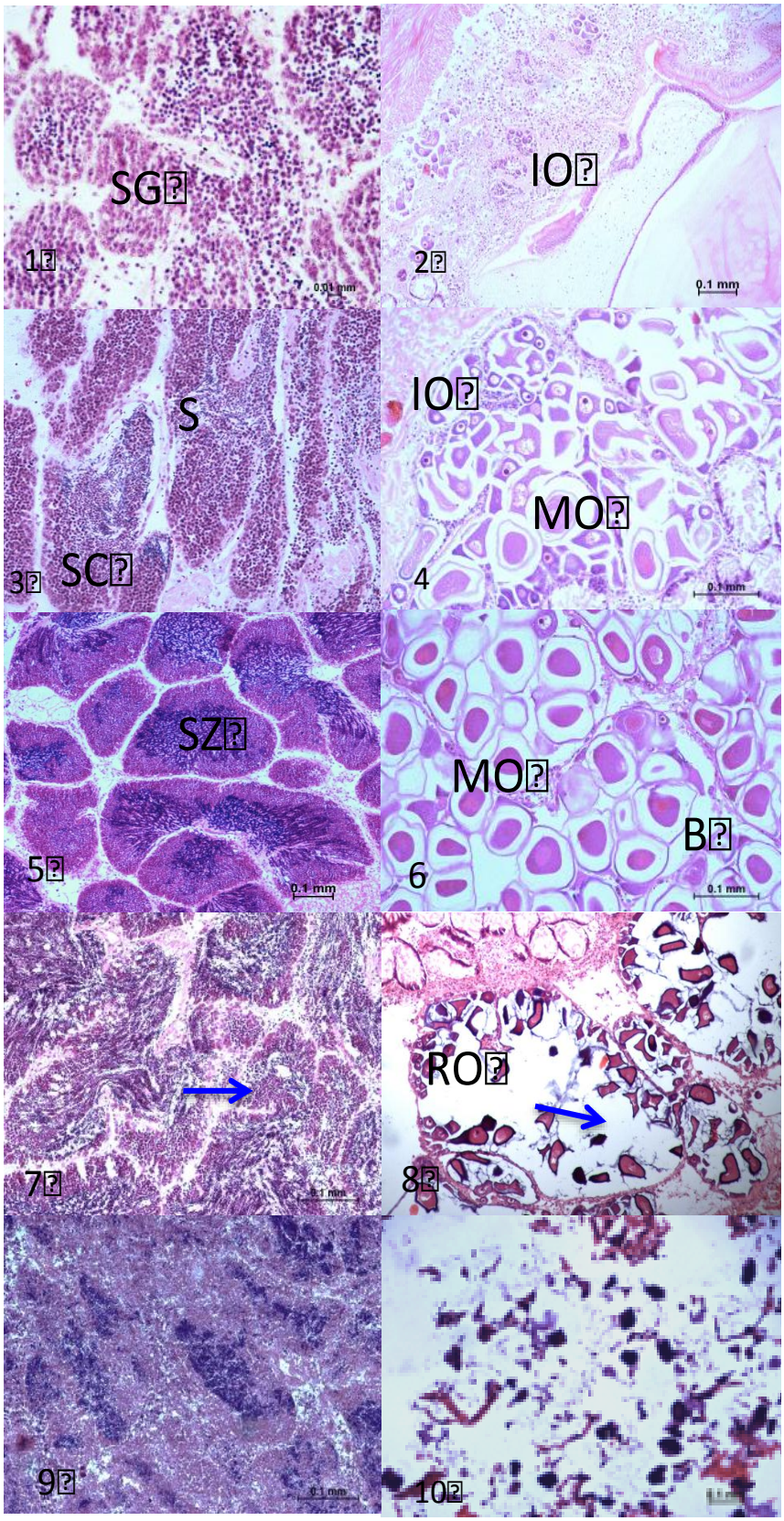
SG
1
IO
2
0.1 mm
S
SC
3
IO
MO
4
0.1 mm
SZ
5
0.1 mm
MO
B
6
0.1 mm
7
RO
8
9
10

Figure 2: Plates of gonads at each stage of gonadal maturity (taken at x 20 magnification, scale bar equals 0.1 mm). 1 male early developing with spermagonia (SG); 2 female early developing with immature oocytes (IO); 3 male late developing with spermatocytes (SC) and spermatids (S); female late developing with immature oocytes (IO) and a small number of mature oocytes (MO); 5 ripe male with spermatids (S) and spermatozoa (SZ) in plug like arrangement; 6 ripe female mature oocytes (O) and thin barriers (B) between oocytes; 7 spawning partially spent male arrow indicates sperm leaving plugs; 8 spawning partially spent female with residual oocytes (RO) and arrow showing empty spaces in gonads; 9 and 10 spent male and females respectively.

Table 3a: Description of male *Mytilus edulis* gonads.

Stage	Description
Early Developing	Small amount of large immature spermatogonia.
Late developing	Greater volume of gonad apparent, comprised of spermatogonia and spermatocytes. Although the sperm is still arranged in a circular fashion, it is less tightly packed than earlier stages.
Ripe	The majority of the tissue section comprised of mature sperm. Mainly spermatozoa, tails of the spermatozoa facing the lumen. Again despite appearing highly organised, less compacted than the previous stage.
Spawning/partially spent	Mature spermatozoa have left the gonad, some loose sperm visible in the tissues, and large spaces becoming apparent in the gonads.
Spent/resorbing	Large spaces clearly visible in the tissues where mature gonads were, the section no longer comprised mostly of gonad. Residual sperm lines the empty gonad plugs, and some free in the tissues.

Table 3b: Description of female *Mytilus edulis* gonads.

Stage	Description
Early developing	Small number of oocytes in pockets throughout the section with none free in the lumen.
Late developing	Pockets of oocytes have increased in size, as have the number and size of oocytes. Borders between each oocyte more visible now.
Ripe	The majority of the section is composed of oocytes, they are arranged in a net like complex. They are larger and no longer totally connected to lumen, many appear free.
Spawning/partially spent	The net - like pattern has disintegrated, most oocytes have been released or are free in the tissues. Those that remain may look broken. No trace of net like pattern remains.
Spent/resorbing	Large spaces, with small amounts of broken resorbing oocytes remaining.

2.9 *Pathology*

The health of all bivalves was assessed using histology. This method was chosen as using this particular technique the sex and reproductive maturity in addition to any pathologies or parasites can be noted. By using histology any pathologies or parasites can be identified e.g. neoplasia, ciliates, gregarines. However due to the nature of histology where a section of the animal is taken there can be an underestimation of the parasite intensity and trematode species cannot be identified accurately unlike when using squash preparations. The presence and intensity of any parasites present was recorded. If neoplasia was diagnosed it was graded according to a scale from Collins and Mulcahy, (2003) (Table 4). Each tissue section per animal was processed in the same manner – a grid was drawn around the section to be examined. Examination of the slides began in the top right hand corner and moved across until the final grid line was in view, then the field of vision was moved down and moved across from the left-hand side. This was repeated for all slides, however if sporocysts were present they were counted at 20x magnification. Only intact sporocysts were counted not those that were releasing cercariae.

Table 4: Description of severity of neoplasia stages (Collins and Mulcahy, 2003).

Stage	Description
0	Absence of neoplastic cells from the tissue section
1	Appearance of low numbers of neoplastic cells in connective tissue underlying the digestive diverticula and gonads.
2	Migration of neoplastic cells to connective tissues away from the digestive and gonadal tissues.
3	Presence of neoplastic cells in all tissue types of individual and within haemolymph sinuses.
4	Large concentrations of neoplastic cells throughout the body.

(a.) (b.)

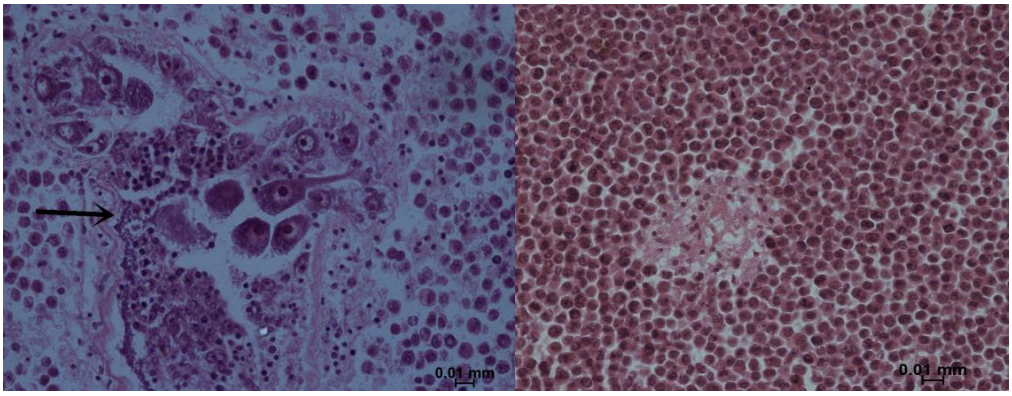

Figure 3: Disseminated neoplasia in the cockle *Cerastoderma edule*, showing (a.) the early stages of the disease and (b.) the latter stages of disseminated neoplasia (x40 magnification, scale bar = 0.01 mm).

(a.) (b.)

(c.) (d.)

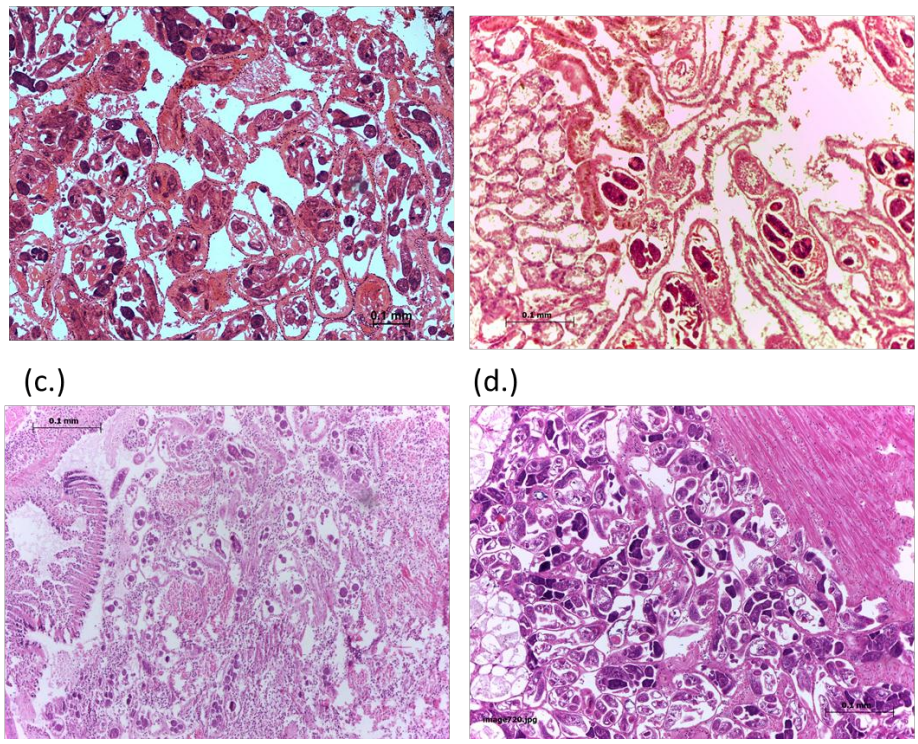

Figure 4: Trematode sporocysts (a.) *Labratrema minimus* in *Cerastoderma edule* (b.) *Bucephalus* spp. in *Mytilus edulis* (c.) *Meiogymnophallus minutus* in *Scrobicularia plana* and (d.) *Meiogymnophallus strigatus in Angulus tenuis*. (Magnification x 20, scale = 0.1 mm).

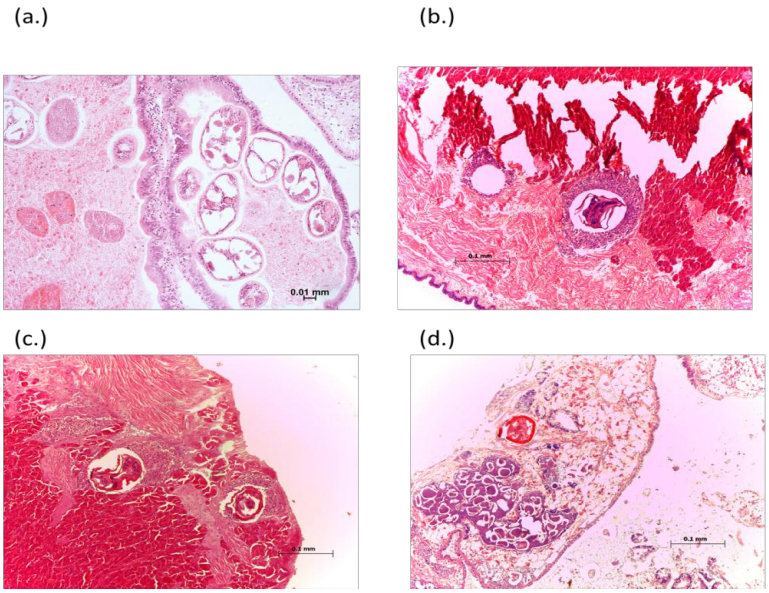

Figure 5: Trematode metacercariae identified in the study (a.) *Meiogymnophallus minutus* in *Cerastoderma edule* (scale bar = 0.01 mm), (b.) *Himasthla interrupta* in *Mytilus edulis* (scale bar = 0.1 mm) (c.) *Himasthla elongata* in *Mytilus edulis* (scale bar = 0.1 mm) (d.) *Gymnophallus choleduchus* in *Mytilus edulis* (scale bar = 0.1 mm).

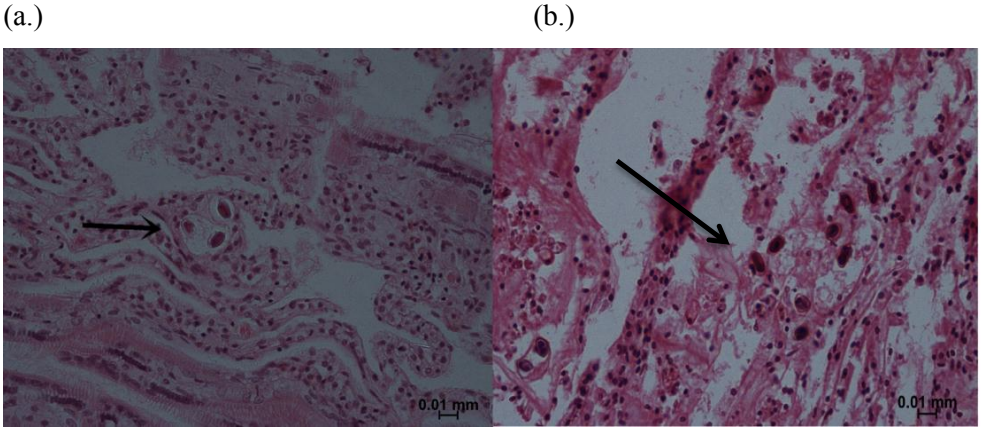

Figure 6: *Nematopsis* sp. oocysts in *Cerastoderma edule* (magnification x 40, scale bar = 0.01 mm).

(a.) (b.)

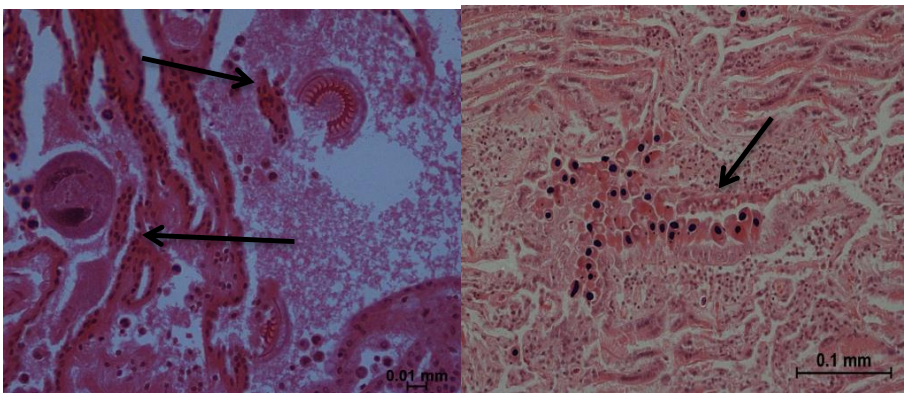

Figure 5: (a.) *Trichodinia*-like ciliates (magnification x 40, scale bar = 0.01 mm) and (b.) *Rynchodida*-like ciliates in the cockle, *Cerastoderma edule* (magnification x 20, scale bar = 0.1 mm)

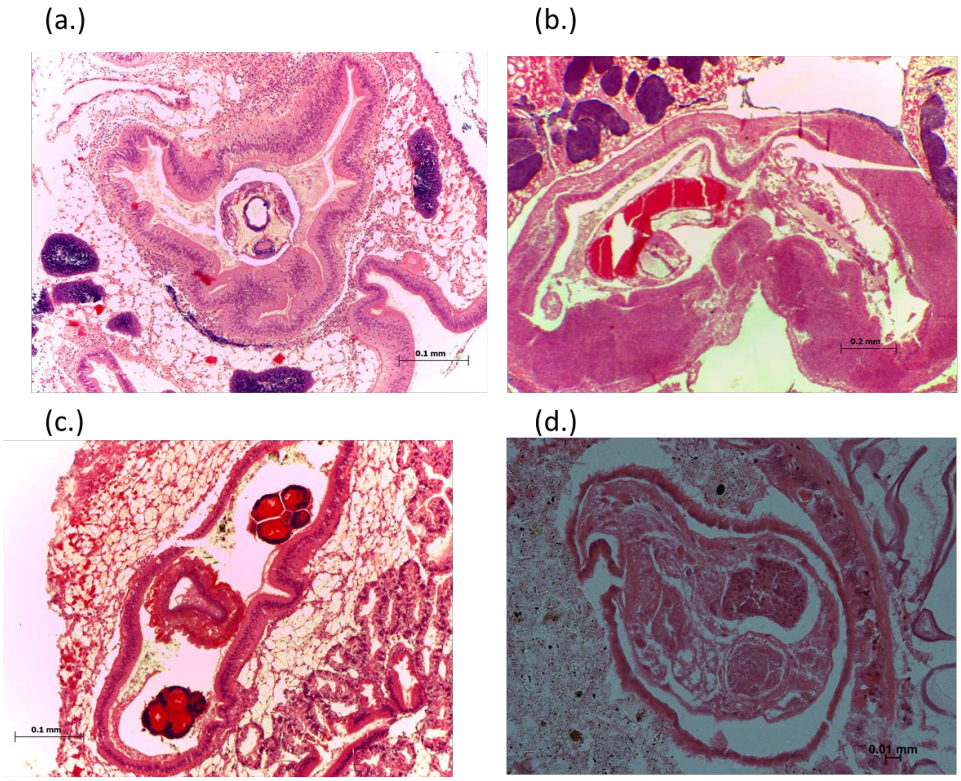

Figure 6: (a.) Cestode in *Mytilus edulis* (scale bar = 0.1 mm) (b.) *Mytilicola intestinalis* in *Mytilus edulis*, (scale bar = 0.2 mm) (c.) Copepod in *Mytilus edulis*, (scale bar = 0.1 mm) (d.) *Paravortex cardii* in *Cerastoderma edule* (scale bar = 0.01 m).

2.10 *Molecular biology*

2.10.1 *DNA Extraction*

Gill and adductor muscle from all animals were frozen and retained for PCR. DNA was extracted using a Chelex (10 %) extraction (Walsh *et al*., 1991). Samples of bivalves from June, July and August 2010 and June 2011 from both sites were screened using C2/C6 primers for ostreid herpes virus and its variants) as it has been found in oysters from Bannow Bay previously (Lynch *et al*., 2012) (Renault *et al*., 2001) and universal primers for the amplification of 18s rDNA region (Zhang *et al*., 2005) which is routinely used for aquatic microbiology studies. Double distilled water (ddH_2O) was used as a negative control (n = 2) in each reaction and ostreid herpes infected oyster tissue was used a as a positive control.

2.10.2 Ostreid herpes virus and its variants

The PCR reaction mix was equal to 50 µl and consisted of (for one individual); 10 µl Green Go Taq™ Reaction Buffer (PROMEGA™), 5 µl dNTPs, 0.5 µl MgCl2 , 1 µl C2 primer, 1 µl C6 primer, 0.5 µl Taq DNA Polymerase (Advanced Bioltechnologies) and 1 µl bivalve DNA. Amplifications were carried out using a Hybaid thermocycler, the reaction cycle was as follows: 94 °C for two minutes, 35 cycles of 94 °C for one minute, 50 °C for 1 minute and 72 °C for one minute, with a final annealing stage of 72 °C for five minutes (Table 5).

2.10.3 18s rDNA region

The PCR mix equaled 25 µl, for one individual the reaction mix was composed of: 5 µl Green Go Taq™ Reaction Buffer (PROMEGA™), 2.5 µl dNTPs, 2 µl MgCl2 , 0.5 µl forward primer, 0.5 µl reverse primer, 0.2 µl Taq DNA Polymerase (Advanced Biotechnologies) and 1 µl bivalve DNA. Amplifications were carried out using a Hybaid thermocycler, the reaction cycle was as follows: 95 °C for five minutes, 35 cycles of 95 °C, 55 °C and 72 °C for 30 seconds each, the final step had a duration of 10 minutes and a temperature of 72 °C (Table 5).

Any resultant PCR products for all were then visualised on a 2% agarose gel after electrophoresis. There were no bands on any negative controls.

Table 5: Primers used for molecular screening.

Name		Sequence	Reference
Ostreid Herpes	C2	5'CTCTTTACCATGAAGATACCCACC3'	Renault *et al*., (2001)
	C6	5'GTGCACGGCTTACCATTTTT3'	
Universal	18ScomF1	5'GCTTGTCTCAAAGATTAAGCCATGC3'	Zhang *et al*., (2005)
	18ScomR1	5'CACCTACGGAAACCTTGTTACGAC3'	

2.11 *Statistics*

2.11.1 *Cockle reproduction (Chapter 3)*

Data were analysed using MINITAB ® Release 14 and PASW Statistics 17 statistical software. Normality was determined using a Kolmogorov-Smirnov Test. A Mann Whitney test was used to determine if there was a significant difference between the mean temperature, mean length, mean whole, tissue and shell weights, or condition index between the sites. A Kruskal-Wallis non-parametric test was used to assess if there was a significant difference between monthly condition indices and a Pearson product moment correlation was applied to see if there were correlations between condition index and tissue weight or temperature. A Spearman rank order correlation was used to determine if there was an association between temperature and reproduction. A $\chi 2$ test, with the Yates correction, was utilised to ascertain if the sex ratio adhered to a 1:1 ratio.

2.11.2 *Mussel reproduction (Chapter 4)*

PASW Statistics 17 statistical software was used to analyse the data. Normality was determined by a Kolmogorov-Smirnov Test. A Mann-Whitney test was used to see if there was a significant difference between the mean monthly temperatures at both sites. An Independent samples t-test was performed to verify if there was a significant difference between mussel length, width, height, weights and condition index between the sites. The Pearson product-moment correlation was used to see if there was a correlation between sea temperature and condition index. A χ^2 test with the Yates correction applied was utilised to ascertain if the sex ratios at both sites differed significantly from a 1:1 ratio.

Statistical analysis was performed using IBM®SPSS® Statistics version 20 for the other bivalves sampled (*Macoma balthica*, *Angulus tenuis* and *Scrobicularia plana*).

Normality was tested using a One-Sample Kolmogorov-Smirnov test and homoscedasticity was determined using a Levene's Test for Equality of Variances. An Independent samples t-test was used to determine significant difference if any between length, width, height, whole weight, shell weight, tissue weight and parasite prevelance and intensity between sites.

2.11.3 *Cockle pathology (Chapter 5)*

Statistical analysis was performed using IBM®SPSS® Statistics version 20. Normality was tested using a One-Sample Kolmogorov-Smirnov test and homoscedasticity was determined using a Levene's Test for Equality of Variances. Firstly significant differences between surfaced and buried cockles were assessed at each site. An Independent Samples T-test was used to determine if there was a significant difference between the prevalence of sporocysts and metacercariae, metacercariae, the intensity of *Nematopsis* sp. oocysts, and the prevalence and intensity of *Rynchodida*-like ciliates, *Trichodinia*-like ciliates and *Paravortex cardii*. It was also utilized to determine the significant difference if any between prevalence and intensity of metacercariae, fungal infection, *Rickettsia*-like organism, and unidentified bacteria 1. Mann Whitney tests assessed the significant difference between fungal infections in Bannow Bay, *Rickettsia*-like organisms in Flaxfort Strand, and tissue damage (broken/melanised gills, digestive area, gonads). The significant difference if any between temperature, prevalence, and intensity of neoplasia and prevalence of sporocysts and *Nematopsis* sp. oocysts was evaluated using a Mann Whitney test. Correlations between temperature and neoplasia and other parasites were judged using a Pearson product moment correlation. Chi square tests, with a Yates correction factor were used to see if there was a significant difference between males and females with disseminated neoplasia and parasites.

Secondly non-metric multi-dimensional scaling (NMDS) analysis was performed, using PRIMER 6, to determine if there were patterns in parasites and morphological changes across several factors and the intensity of infection was used. NMDS was chosen as it can be used with non-normal and data with many zeros (McCune and Grace 2002). The goodness-of-fit associated with each NMDS is given by a stress value, which is obtained as the regression of the interpoint distances from the plot on the corresponding dissimilarities (Clarke and Warwick 1994). NMDS ranks points in low-dimensional space such that the relative distances apart of all points are in the same rank order as the relative dissimilarities of the samples (Clarke and Gorley

2006). The ordination is based on a Bray-Curtis dissimilarity matrix and all data were log (x +1) transformed prior to use. Analysis of similarity (ANOSIM) was performed comparing the categories within each factor.

2.11.4 *Mussel pathology (Chapter 6)*

Statistical analysis was performed using IBM®SPSS® Statistics version 20. Normality was tested using a One-Sample Kolmogorov-Smirnov test and homoscedasticity was determined using a Levene's Test for Equality of Variances. An Independent Samples t-test was used to assess whether there was a significant difference between the prevalence of fungal growths, granulomas, lipofuscin granules in the mantle, lipofuscin granules in the organs, ciliates and *Ancistrum mytili* between the two sites. The same test was used to compare if there was a significant difference in the intensity of *Mytilicola intestinalis* and ciliates when contrasting the two sites. A non-parametric Mann Whitney U test was utilized to evaluate if there was a significant difference in the prevalence of metacercariae, *Nematopsis* sp., *Mytilicola intestinalis*, and unknown species of copepod, *Rickettsia*-like organisms, eosinophilic cells, haemocyte infiltration, gill damage, and digestive tubule damage between Bannow Bay and Flaxfort Strand.

Non-metric multi-dimensional scaling (NMDS) analysis was performed, using PRIMER 6, to determine patterns in parasites across several factors (site, sex, reproductive maturity, month and temperature). Several categories of data were pooled for the NMDS analysis and designated as variables - these were: digenean trematodes (all reproductive stages), all copepod species and ciliates. NMDS was chosen as it can be used with non-normal and zero rich data (McCune and Grace 2002). The goodness-of-fit associated with each NMDS is given by a stress value, which is obtained as the regression of the interpoint distances from the plot on the corresponding dissimilarities (Clarke and Warwick 1994). NMDS ranks points in low-dimensional space such that the relative distances apart of all points are in the same rank order as the relative dissimilarities of the samples (Clarke and Gorley 2006). The ordination is based on a Bray-Curtis dissimilarity matrix and the data were log (x+1) transformed prior to use in Primer 6. Analysis of similarity (ANOSIM) was performed comparing the categories within each factor e.g. between males, females and indeterminate sex using PRIMER 6 too. Mussels with no parasites/pathologies were excluded, as were parasites that were in less than 5 % of the data e.g. the one individual with sporocysts as it created a skew in the data.

Comparison of other bivalves was performed using IBM®SPSS® Statistics version 20. Normality was tested using a One-Sample Kolmogorov-Smirnov test and homoscedasticity was determined using a Levene's Test for Equality of Variances. Mann Whitney U tests were used to determine if there was a significant difference in the prevalence of neoplasia, prevalence of trematodes, prevalence of *Nematopsis* sp., intensity of *Nematopsis* sp., prevalence of *Mytilicola intestinalis*, *Rickettsia*-like organisms and the intensity of *Ancistrum mytili*. Independent samples t-test was utilized to ascertain the significant difference if any between intensity of trematodes, intensity of *Mytilicola intestinalis*, and the prevalence of fungus infection and *Ancistrum mytili*

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CHAPTER 3: CLIMATE CHANGE IMPACTS ON POTENTIAL RECRUITMENT IN AN ECOSYSTEM ENGINEER.

Morgan, E., O'Riordan, R.M., Culloty, S.C., (2013). Climate change impacts on potenitial recruitment in an ecosystem engineer. Ecology and Evolution, 3(3):581-594. Unmodified version.

3.1 ABSTRACT

Climate variability and the rapid warming of seas undoubtedly have huge ramifications for biological processes such as reproduction. As such, gametogenesis and spawning were investigated at two sites over 200 km apart on the south coast of Ireland in an ecosystem engineer, the common cockle, *Cerastoderma edule*. Both sites are classed as Special Areas of Conservation (SACs) but are of different water quality. *Cerastoderma edule* plays a significant biological role by recycling nutrients and affecting sediment structure, with impacts upon assemblage biomass and functional diversity. It plays a key role in food webs, being a common foodstuff for a number of marine birds including the oystercatcher. Both before and during the study (early 2010 – mid 2011) Ireland experienced its two coldest winters for 50 years. Since the research demonstrated only slight variation in the spawning period between sites, despite site differences in water and environmental quality, temperature and variable climatic conditions were the dominant factors controlling gametogenesis. The most significant finding was that the spawning period in the cockle extended over a greater number of months compared to previous studies and that gametogenesis commenced over winter rather than in spring. Extremely cold winters may impact on the cockle by accelerating and extending the onset and development of gametogenesis. Whether this impact is positive or negative would depend on the associated events occurring on which the cockle depends i.e. presence of primary producers and spring blooms, which would facilitate conversion of this extended gametogenesis into successful recruitment.

Keywords: Phenology, climate variability, *Cerastoderma edule*, condition index, gametogenesis, ecosystem engineer.

3.2 INTRODUCTION

It is evident that changes are ongoing in the marine environment in terms of temperature, wind patterns and sea levels orchestrated by climate change, with the swift nature of such changes causing much concern (Philippart *et al*., 2011). These changes are being seen globally, but not equally and the seas around Europe are disparately affected and were included as “rapid warming” seas in recent studies of warming in large marine ecosystems (Belkin, 2009; Philippart *et al*., 2011). The period 1965–2004 in the North Atlantic has been

a time of net heat accumulation and yields from European fisheries have also shown changes, with decreases in the Celtic Biscay Sea area but increases in the Norwegian Seas (Philippart *et al*., 2011). Trends in the Irish Sea indicate warming of sea surface temperatures by 0.5 to 1 °C from 1870–2007, as well as a tendency for increases in air temperature, sea level and acidification (UKMMAS 2010). The sea surface temperature around the UK and Ireland has increased at 6 times the rate of the global average (Frost *et al*., 2012). For the preceding 30 years there have been increases of 0.2 – 0.6 °C in sea temperature per decade around the UK and Ireland and the mean surface ocean pH has reached 8.14 in comparison to 8.25 in the 18th century (Heath *et al*., 2012). The mean annual air temperature recorded in Ireland has increased by 0.7°C in the period 1890 – 2004 and the northern hemisphere has been warmer since 1980 than at any time in the last 2000 years (Philippart *et al*., 2011). However, in 2010 Ireland experienced its coldest winter in 50 years (www1), related to the negative phase of the North Atlantic Oscillation (NAO), whose variability could be related to anthropogenic warming (Goodkin *et al*., 2008). It is predicted that sea levels around Ireland will increase by 18 – 59 cm in the 21st century, and water quality is expected to be impacted deleteriously by the re-entry of contaminants that had previously been sediment-bound in lowlands, and estuaries during extreme events that generate run off (Schiedek *et al*., 2007). As a result, species, which have small thermal tolerance windows, will be jeopardised (www 2).

Cerastoderma edule is a common infaunal bivalve whose range spans from the Barents Sea to West Africa and is less commonly found in the southwest Mediterranean (Reise, 2003; Cardoso *et al*., 2009). Preferred habitats of *C*. *edule* include sandy bays and estuaries but it can also be found in gravel and mud (www3; André *et al*., 1999; Reise, 2003). Cockles can comprise up to 60% of the biomass of an area (Dabouineau and Ponsero, 2009) and they play an important role in the ecosystem (Figure 1). They are a link between primary producers (plankton) and higher trophic levels as cockles are preyed upon by crabs e.g. *Carcinus maenas*, shrimp e.g. *Crangon crangon*, fish e.g. *Pleuronectes platessa* and birds e.g. *Haematopus ostralegus*. Indeed *C*. *crangon* and *P*. *platessa* may consume up to 90% of juvenile cockles in an area (Cesar, 2009). *Carcinus maenas*, *C*. *crangon* and *P*. *platessa* are themselves subject to fisheries, as is the cockle and therefore create a link between marine and terrestrial food webs, in addition to being commercially important. Cockles themselves provide a habitat for up to 16 species of digenean trematode (de Montaudouin *et al*., 2009),

turbellaria and various other parasites, acting as a first and second intermediate host. Cockles too are responsible for a range of other ecosystem services such as: recycling nutrients, acting as a carbon store, cycling energy and increasing ammonium concentrations which in turn increases primary producers. They add to the overall diversity of an area and it is thought that increased biodiversity may increase ecosystem resilience and by their movements in the sediment they facilitate the exchange of dissolved nutrients to the water column and may increase the depth to which oxygen dissipates into the sediment (Cesar, 2009). It has been suggested that estuarine and coastal areas are prime candidates for the study of climate change as they are shallow, and alterations in thermal regimes will be readily apparent (Moore *et al*., 2011; Madeira *et al*., 2012). Therefore cockles were identified as an ideal candidate to evaluate the effects of climate variability on biological processes and in the case of this study: reproduction.

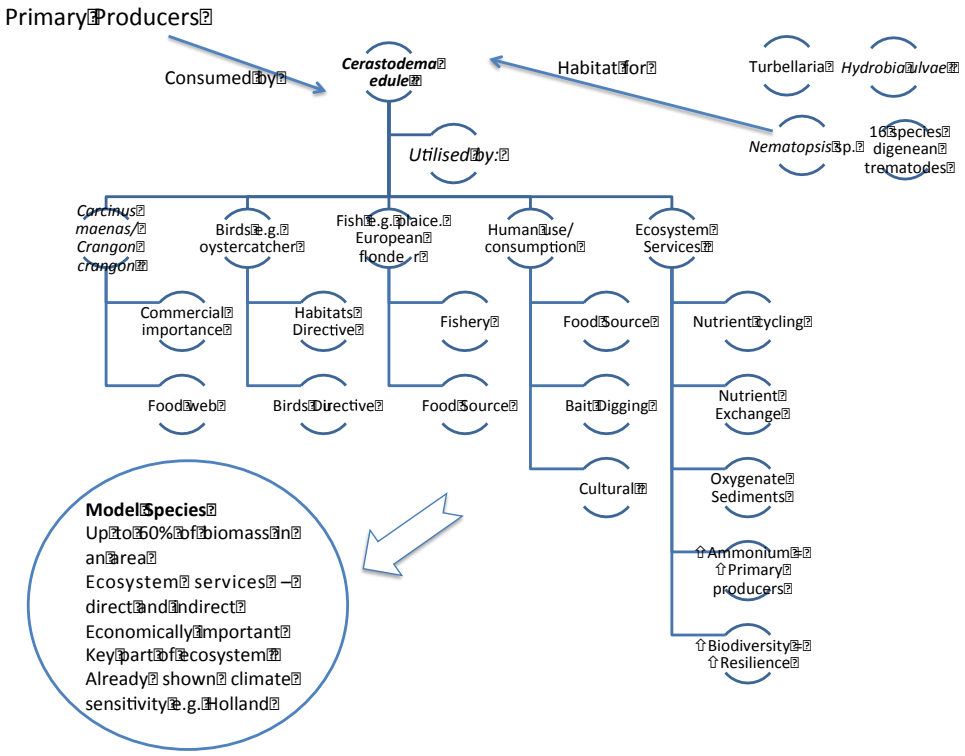


Figure 1: Simplified diagram of ecosystem including services of cockles in their habitats showing its appropriateness as a model species for climate variability studies.

Previous research has shown that recruitment in cockles can vary markedly in success between years and sites, with large increases in density occurring over a short period of time (Ramon, 1996; Honkoop *et al*., 1998; Sauriau and Kang, 2000; Cardoso *et al*., 2009). Although cockles are known to be sensitive to colder conditions, experiencing mortalities during cold spells (Ramon, 1996; Reise, 2003; Strasser *et al*., 2001; Flach, 2003; Tyler-Walters 2007) greater recruitment has been seen in many areas e.g. Burry Inlet, following severe winters (Yankson, 1986; André and Rosenberg, 1991; Lindegarth *et al*., 1995). A decline in recruitment among *C. edule* and other bivalves (*Mytilus edulis*, *Mya arenaria*, *Macoma balthica*) in the Dutch Wadden Sea and Sweden have been linked to warming winters for the previous 15 years (Cardoso *et al*., 2009). It has been shown that following warmer winters smaller, less viable eggs, with less lipids, are produced (Cesar, 2009) and gametogenesis can extend further in the year and not allow for a sufficient rest period for

recovery, prior to the beginning of the next reproductive cycle (Guillou and Tartu, 1992). If conditions for optimal spawning (cold winters) are rarer, short-lived species, such as the cockle, may not experience them in their life time (Cardoso *et al*., 2009). This could lead to an overall decrease in cockle biomass, which would have serious ramifications for not only cockles, but the ecosystem as a whole, because they as previously stated, are an integral part of their habitats.

Cerastoderma edule is a dioecious, broadcast spawner (Boyden 1971; Dauboineau and Ponsero, 2009); therefore fertilisation in cockles is external, with males and females producing gametes, which are released into the water column (Lindegarth *et al*., 1995; André *et al*., 1999). It is an income breeder, acquiring energy as it produces gonads. Growth of cockle planktonic larvae proceeds quickly, and they persist in the water column for at least 2 - 3 weeks, but perhaps up to 5 weeks (Boyden, 1971; Lindegarth *et al*., 1995; Honkoop and van der Meer, 1998; André *et al*., 1999; Bouma *et al*., 2001; Reise, 2003). Sexual maturity is usually reached when cockles are 18 months old or of shell length of 15 - 20 mm (Seed and Brown, 1977; Dabouineau and Ponsero, 2009) but Cardoso *et al*. (2009) recorded gonads in cockles > 12 mm in shell length in cockles from the Dutch Wadden Sea. Settlement of cockles at Strangford Lough, Ireland has been described in August and September (Seed and Brown, 1977) but in France between September and October (Guillou and Tartu, 1994). External parameters such as temperature are known to affect gametogenesis and onset of spawning in bivalves; (Gosling 2003; Drummond *et al*., 2006). Temperature acts to synchronise internal processes in bivalves, such as nutrient storage and the endocrine system (Brown, 1984).

The aims of this study were to examine the reproductive cycle of *Cerastoderma edule*, using it as a model species to assess the effects of climate variability on an r – selected (small body size, early maturity, high fecundity, short generation time), ecosystem engineer, which is integral to food webs, provides habitat for other organisms and has commercial importance. The study was undertaken at two sites which are classed as Special Areas of Conservation – SACs which are areas created under Article 3 of the EU Habitats Directive, and their purpose is to aid in the conservation of species and habitats on Annex I and Annex II of the Habitats Directive, by having conservation sites of good quality (www4; www5; www6). Two sites were chosen as great variation can be seen in cockles separated by a few metres on the same

bed – so this study aimed to determine if there was a general pattern apparent in reproduction (e.g. initiation of gametogenesis/spawning), despite local variation (e.g. in temperature, water quality) by examining two sites at the same latitude but 215km apart. Cockle reproduction has been assessed previously, but rarely using histology, the previous study performed in Ireland was in 1977, and the last study published from Europe was in 1989 and used gamete volume fraction. So a histological study was deemed appropriate, which, although labour intensive, gives a much clearer picture than just assessing presence of larvae in the plankton etc. The alterations in Ireland's weather patterns and climate, due to possible climate change, were especially apparent during this study with two extremely cold winters, the coldest in 50 years (www 5), therefore this offered a unique opportunity to evaluate the effects of an unusually cold winter on gametogenesis and recruitment in the cockle after a period of prolonged warming.

3.3 MATERIALS & METHODS

Figure 2: Map of Ireland plus study locations Bannow Bay and Flaxfort Strand with additional temperature stations included (aerial photos taken at 500 m). 1. Johnstown Castle, 2. Bannow Bay, 3. Cork Airport, 4. Flaxfort Strand, 5. Bantry Bay.

3.3.1 *Study sites*

Two sites 215km apart were selected. Flaxfort Strand (N51°38' 47.4", W 008°41'45.0") is a small semi-exposed bay on the south west coast of Ireland (Figure 2). It is composed primarily of sand flats and extensive colonisation by green algae of *Enteromorpha* spp. can be seen on the upper shore during the summer months. The tidal range is 4.2 m. The River Argideen drains into the area and the water quality is classed as eutrophic by the Environmental Protection Agency (www5). Land usage of the surrounding areas is predominantly dairy farming. Flaxfort Strand is part of Courtmacsherry Bay, which is designated as a Special Area of Conservation (SAC: 001230). It was allocated this status as ten habitats recorded on Annex 1 of the EU Habitats Directive (www7) make up this bay. Clonakilty Bay which is adjacent is classed as a Special Protected Area (SPA: 004081); it includes habitats classed as Annex 1 on the EU Habitats Directive (www8) plus bird species with Annex 1 status on the EU Birds Directive e.g. bar tailed godwits, *Limosa lapponica* and golden plover, *Pluvialis apricaria* frequent this area.

Bannow Bay (N52°13'07.9", W006°46'37.8") is located on the south east coast of Ireland; it is a shallow bay that is over ten times larger than Flaxfort Strand. It has an area of ~1050 hectares and at its greatest is 8km in length and varies between 1 and 3 km wide and had a tidal range of 3.6 m. Water stays in the bay for two tidal cycles and the bay itself is comprised of a mixture of gravel, sand and mudflats (Cotter *et al*., 2010). Unlike Flaxfort Strand, large banks of *Entermorpha* spp. were not observed for the duration of the study and according to the National Parks and Wildlife Service its habitats are of "general good quality" (www4). It is classified as a Special Protected Area – Site Code IE 0004033 under the EU Birds Directive as it too provides habitat for several Annex 1 bird species (the golden plover, bar tailed godwit) and there are two breeding Annex 1 species present, the little tern, and the kingfisher. Under the EU Habitats Directive (92/43/EEC) it is classed as a Special Area of Conservation (Site Code IE000697) as it encompasses 11 Annex 1 habitats as decreed by the Habitats Directive (www4). Furthermore it is also named as a National Heritage Area (NHA Site Code 0000697) and subject to the European Communities (Quality of Shellfish Waters) Regulations S.I No. 268 of 2006 European Communities (Quality of Shellfish Waters) Regulations 2006.

3.2.2 *Environmental parameters*

One Stowaway ®Tidbit ™ Weatherproof and Waterproof Temperature Logger was placed in a channel of water at each site, adjacent to the main collection area. For the duration of the study they were continuously submerged within the channel. The temperature was recorded every 16 minutes from their deployment until the cessation of the study. The data were retrieved using an Optic Base Station/Coupler Kit and analysed with BoxCar®Pro 4.3 Starter Kit software. Additional temperature data for the study period were provided for Bannow Bay by Met Éireann from Johnstown Castle (20.9 km away) (air temperature) and for Flaxfort Strand by the Marine Institute from Bantry Bay (distance of 65.5 km) (water temperature) and Met Éireann from Cork airport (40.5 km from Flaxfort Strand) (air temperature) (Figure 2).

3.3.3.*Sampling of cockles*

The same methodology was employed at both sites and fieldwork was carried out at monthly intervals commencing in March 2010 in Bannow Bay and April 2010 in Flaxfort Strand, continuing until June 2011 at both sites. 30 buried cockles (buried to a depth of 200– 300 mm in the sediment) were collected monthly from each site at low tide. Cockles in standing water or those that had surfaced were not sampled for this study, only those that remained buried at low tide were included. Due to adverse weather conditions cockles were not collected from Bannow Bay in December 2010.

3.3.4 *Morphometrics and age structure*

The whole weight, shell weight, and tissue weight were weighed to two decimal places (g). The height of each cockle was measured from the umbo to the lip of shell, the length across the widest part of the shell and the width to 1 decimal place in mm. Finally the number of growth rings on each cockle was counted.

3.3.5 *Condition Index*

The mean condition index was calculated on a monthly basis. The average wet tissue weight and average wet shell weight were used as per an altered version of Drummond *et al*. (2006) and Cotter *et al*. (2010) (as they used dry weights).

$$\frac{Condition\ Index\ =\ Wet\ Tissue\ Weight\ (g)\ x\ 100}{Shell\ Weight\ (g)}$$

3.3.6 *Histology*

In the laboratory, cockles were opened carefully and 10 mm transverse sections through the gonad regions were preserved for histology in Davidson's Solution (Shaw and Battle, 1957; Howard *et al*., 2004), and refrigerated for two days at 4˚C. They were sectioned at 5µm and stained with Haematoxylin and Eosin.

The slides were examined at 4x, 10x, and 40x magnification. Sex was determined if gonads were present and gonadal maturity stages were assigned to each of the cockles. Five reproductive stages were identified based on a scale from Drummond *et al*. (2006), which was modified to reflect differences between clam and cockle morphology. These were: early developing, late developing, ripe, spawning/partially spent and spent (Figure 3, tables 1a and 1b). When cockles presented stages that could be described as intermediate between two stages, the stage that dominated the greatest part of the gonads was assigned.

Table 1a: Description of female gonads based on a modified scale from Drummond *et al*. (2006).

Stage	**Description**
Early Developing	Small number of oocytes in discrete areas visible with none free in the lumen. Dark in colour and with grainy borders.
Late Developing	Larger area taken over by oocytes which have increased in size – still some small in size, with a more defined shape. Some now free from the follicle wall, but remaining in discrete pattern.
Ripe	Majority of the section containing oocytes, which are now more uniformly large. Oval in shape and free in the lumen with thin barriers between oocytes. Appear in distinct net pattern.
Spawning/Partially Spent	Broken follicle walls, the number of oocytes reduced. Spaces beginning to show in the gonad region, some oocytes remain that are no longer in a homogenous pattern.
Spent	Section through gonads appears broken, with large spaces evident. Remaining oocytes dark in colour and isolated, with no net-like pattern remaining.

Table 1b: Description of male gonads based on a modified scale from Drummond *et al*. (2006).

Stage	**Description**
Early developing	Gonads have begun to develop, small amount discernible in plugs. Comprised mainly of lighter, rounder and larger spermatogonia encompassing smaller darker spermatocytes. Still appear quite loose in composition.
Late developing	Still mainly spermatogonia and spermatocytes, but some elongating spermatids visible in the centre of the plug. Spermatids stained navy blue in comparison to purple spermatogonia and spermatocytes.
Ripe	Plugs of gonad very organised and intact more compacted than earlier stages. Mainly spermatozoa, tails of the spermatozoa facing the lumen.
Spawning/Partially spent	Spermatozoa leaving plugs, empty spaces visible. The neat organised structure degrading.
Spent	Small amounts of sperm visible scattered through section, follicles broken.

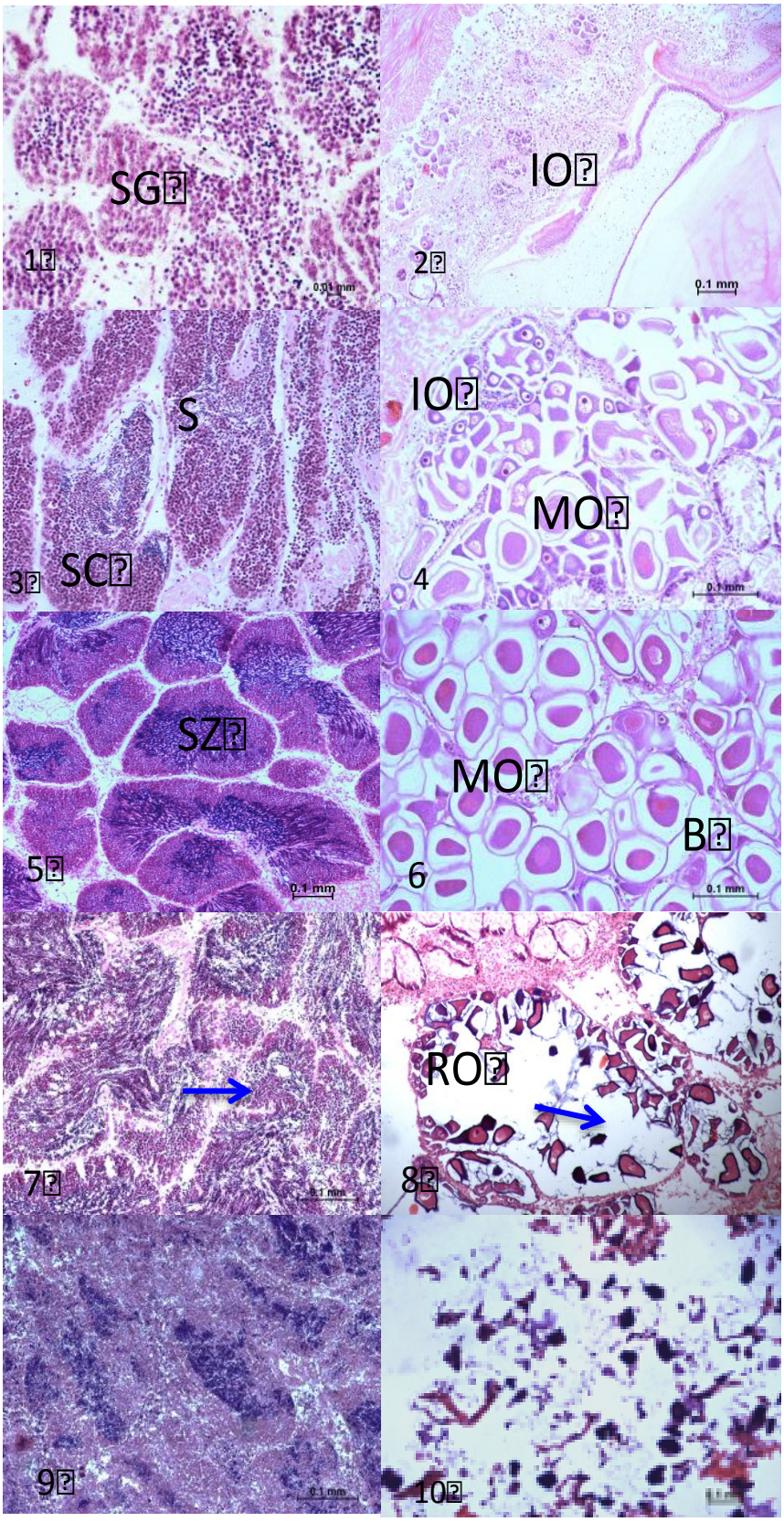

SG
1
IO
2
S
3
SC
IO
MO
4
SZ
5
MO
B
6
RO
7
8
9
10

Figure 3: Plates of gonads at each stage of gonadal maturity (taken at x 20 magnification, scale bar equals 0.1 mm). 1 male early developing with spermagonia (SG); 2 female early developing with immature oocytes (IO); 3 male late developing with spermatocytes (SC) and spermatids (S); female late developing with immature oocytes (IO) and a small number of mature oocytes (MO); 5 ripe male with spermatids (S) and spermatozoa (SZ) in plug like arrangement; 6 ripe female mature oocytes (O) and thin barriers (B) between oocytes; 7 spawning partially spent male arrow indicates sperm leaving plugs; 8 spawning partially spent female with residual oocytes (RO) and arrow showing empty spaces in gonads; 9 and 10 spent male and females respectively.

3.3.7 *Statistical analysis*

Data were analysed using MINITAB ® Release 14 and PASW Statistics 17 statistical software. Normality was determined using a Kolmogorov-Smirnov Test. A Mann Whitney test was used to determine if there was a significant difference between the mean temperature, mean length, mean whole, tissue and shell weights, or condition index between the sites. A Kruskal-Wallis non-parametric test was used to assess if there was a significant difference between monthly condition indices and a Pearson product moment correlation was applied to see if there were correlations between condition index and tissue weight or temperature. A Spearman rank order correlation was used to determine if there was an association between temperature and reproduction. A χ^2 test, with the Yates correction, was utilised to ascertain if the sex ratio adhered to a 1:1 ratio.

3.4 RESULTS

3.4.1 *Temperature data*

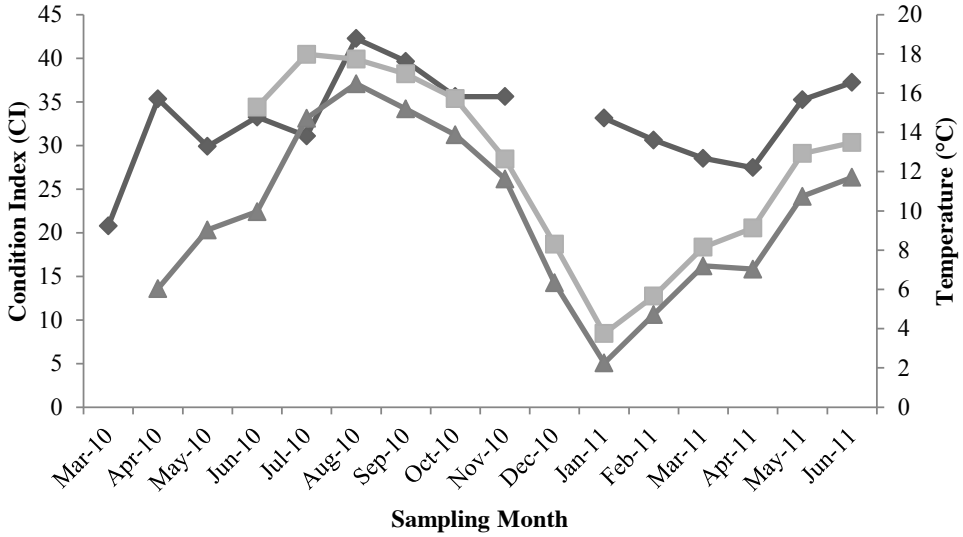
In 2010 the water temperature (recorded using ®Tidbit™) at Bannow Bay ranged from -1.66 °C in December 2010 to 27.97°C in June 2010 with a yearly mean of 13.55°C. For the six months that the temperature probes were deployed in 2011 the mean temperature recorded was 10.74°C and it ranged from -0.44°C in January 2011 to 26.36°C in June 2011. For the whole study period, 2010 and 2011, the temperature range in Bannow Bay was -1.66°C in December 2010 to 27.97°C in June 2010 with an overall mean of 12.34°C. The water temperature in 2010 at Flaxfort Strand ranged from -3.63°C in December 2010 to 26.16°C in

June 2010 with a mean of 13.19°C. The mean temperature recorded from January to June 2011 was 10.53°C, the minimum value recorded was -1.78°C in January and the maximum was 27.44°C in June 2011. Overall for the duration of the study, the minimum temperature recorded was -3.63°C in December 2010 and the maximum was 27.44°C in June 2011. There was no significant difference between the monthly average temperatures between the sites (Mann Whitney test) however mean monthly temperatures measured in 2010 were higher than the corresponding months in 2011.

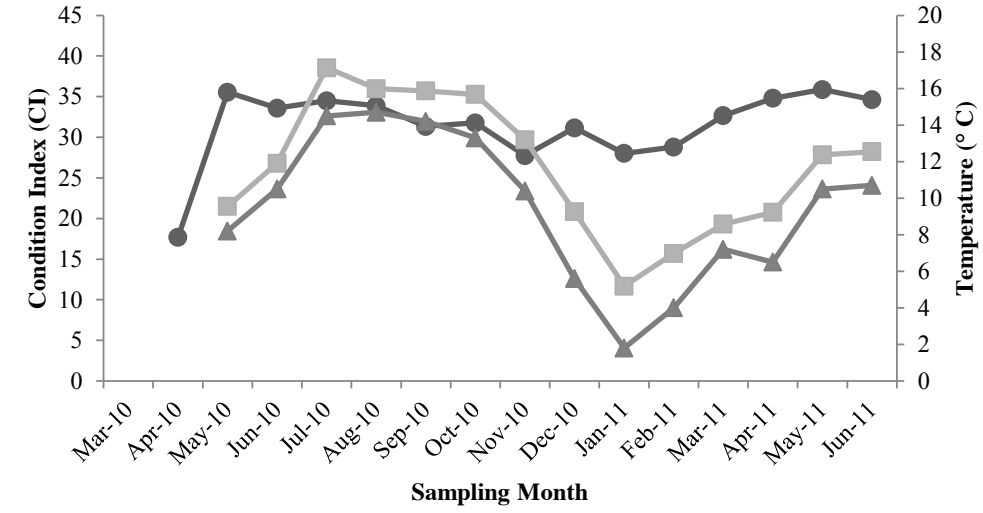
3.4.2 *General Cockle Biology*

Cockles from Bannow Bay were larger in shell length, width and height than cockles from Flaxfort Strand but not significantly so. But the whole weight, shell weight, and tissue weight of cockles from Bannow Bay were significantly greater than those from Flaxfort Strand (Table 2). The mean condition index (CI) of cockles collected from Bannow Bay (33.0 ± 5.28) was greater than the mean condition index of cockles from Flaxfort Strand (31.5 ± 4.63) but not significantly so (Mann Whitney test $P = 0.436$) (Figure 4).

Bannow Bay



Flaxfort Strand



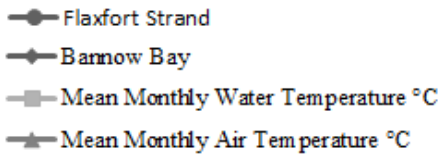
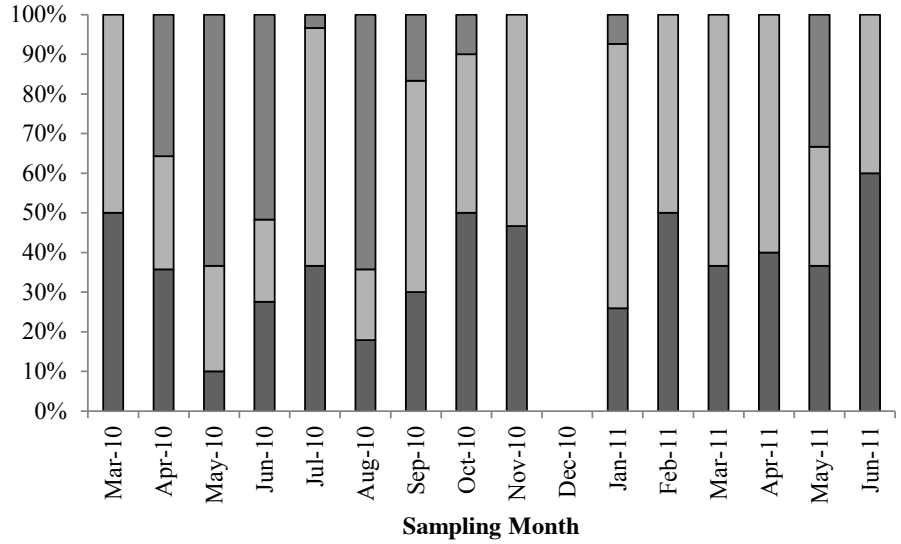


Figure 4: Condition index in cockles collected from Bannow Bay and Flaxfort Strand with mean monthly water and air temperature included.

Also there were a greater number of fluctuations in the CI of cockles collected from Bannow Bay than Flaxfort Strand (which may be related to using wet weight rather than dry or ash free dry weight). There was no correlation between water temperature and condition index for cockles from either of the sites using a Pearson product-moment correlation. Excluding indeterminate cockles the sex ratio at both sites did not differ significantly from a 1:1 ratio; (BB buried ♂ 163: ♀ 195: indeterminate 83: hermaphrodite 0) (FF ♂ 192: ♀ 204: indeterminate 42: hermaphrodite 1) (Figure 5).

Bannow Bay



Flaxfort Strand

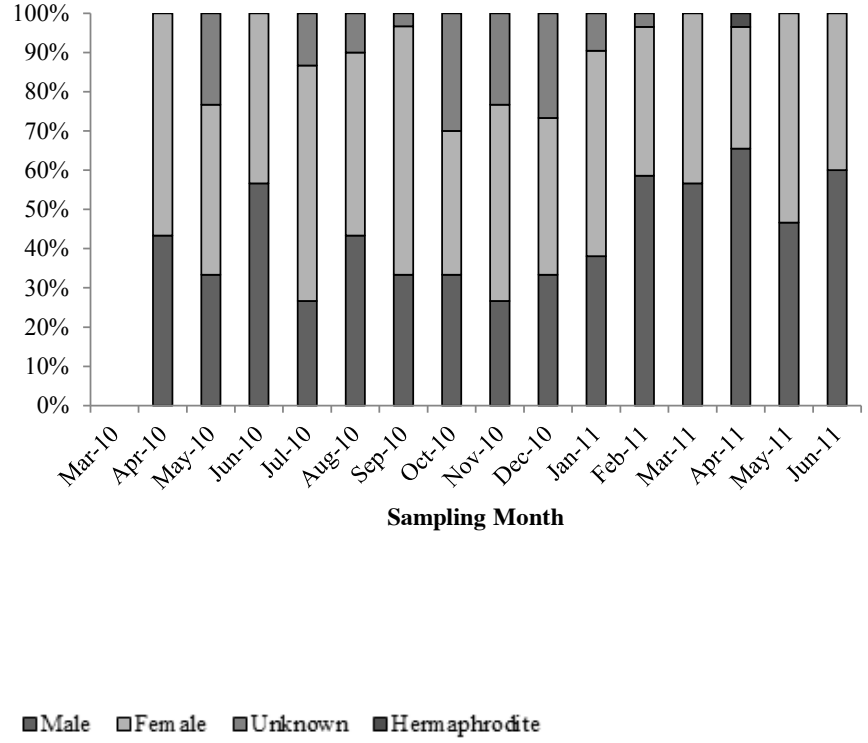


Figure 5: Sex ratio at both sites over the duration of the study period.

The mean number of growth rings of cockles at Bannow Bay and Flaxfort Strand were 2.7 ± 0.84 growth rings and 2.9 ± 0.57 growth rings respectively (Figure 6). There were a significantly greater number of cockles with one or two growth rings at Bannow Bay (n = 193) than Flaxfort Strand (n = 138) (χ^2 – test), while the opposite occurred for three or more growth rings at (Bannow Bay n = 200: Flaxfort Strand n = 277) (χ^2 – test). At both sites the maximum number of growth rings recorded was six.

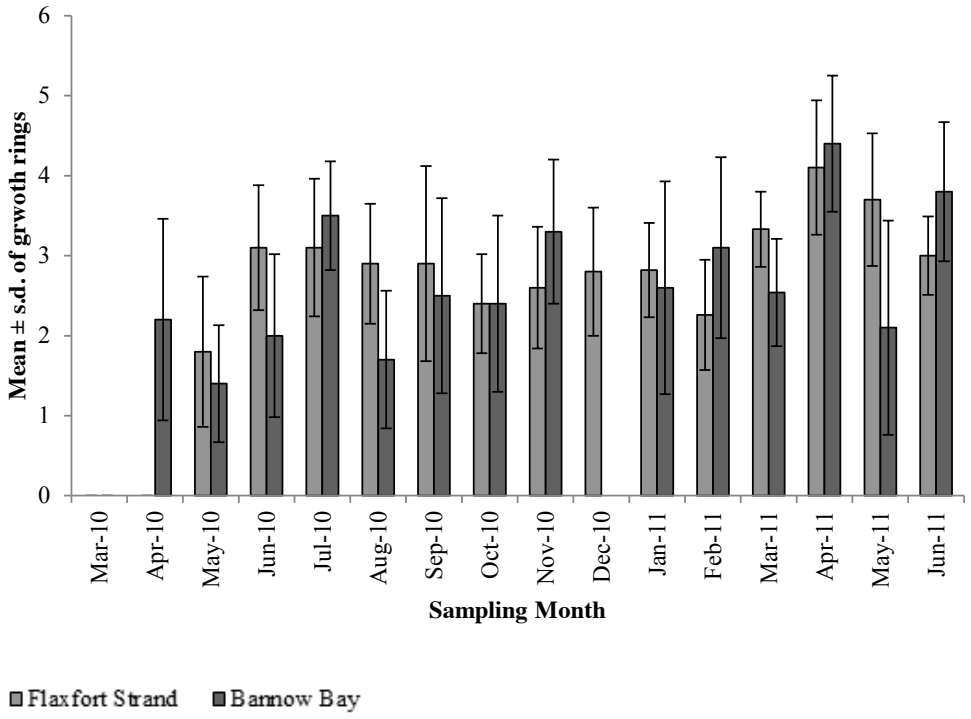


Figure 6: Mean ± s.d. age of cockles per month at Flaxfort Strand and Bannow Bay.

3.4.3 *Staging of Gonadal Maturity*

Five stages of gonadal maturity were observed during the course of the study: early developing, late developing, ripe, spawning/partially spent and spent.

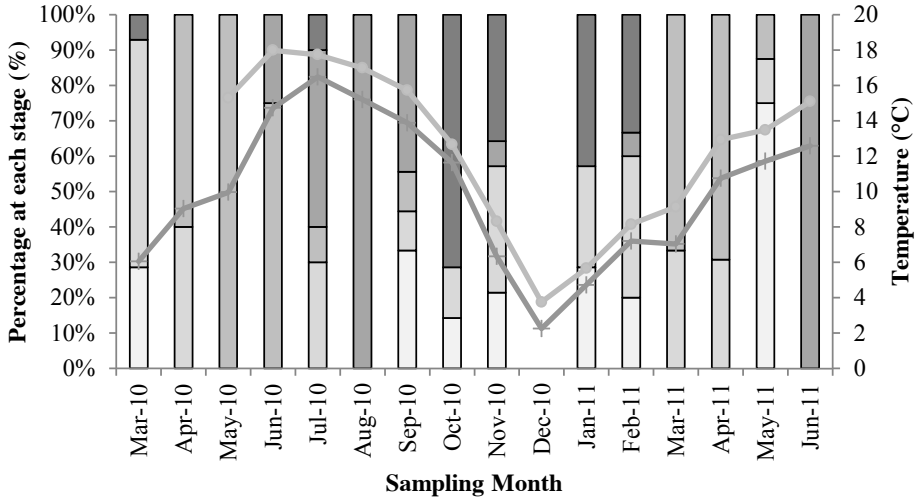
Bannow Bay

In March 2010 upon the commencement of the study at Bannow Bay three stages of gonadal maturity were apparent in males; early developing (28.6%), late developing (64.3%) and spent (7.1%) (Figure 7). Ripe males were recorded from April in 2010 (60%) and spawning lasted from June in 2010 (25%) until November 2010 (7.1%), 100% of males were spawning in August 2010. Gametogenesis began again in September 2010 and early developing males were seen until February 2011 when most cockles were then classified as late developing (42.9%). Ripe males were recorded from March 2011 (66.7%), but spawning did not begin until June 2011 when 100% of sampled males were spawning.

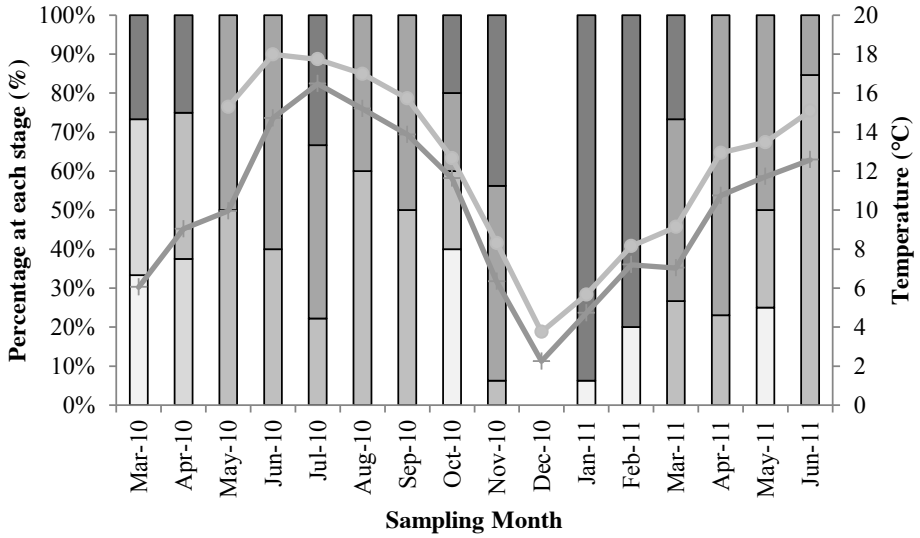
In synchrony with male cockles at Bannow Bay, females in this area were early developing (35.7%), late developing (42.9%) or spent (21.4%) in March 2010. By April 2010, ripe females (37.5%) were observed however some were still late developing (37.5%) and spent (25%). In May 2010 spawning began and like males continued until November 2010. Unlike males, female gametogenesis over winter was not as widespread, and gametogenesis recommenced in most female cockles in January and February 2011. It progressed rapidly to later stages of gonadal development since ripe females were identified from March 2011 (26.7%) when spawning females (46.7%) were recorded too. The peak in spawning was observed in April 2011 (76.9%) decreasing to 50% in May 2011, and by June 2011 the majority of cockles examined were in ripe condition (84.6%).

Bannow Bay

Male



Female



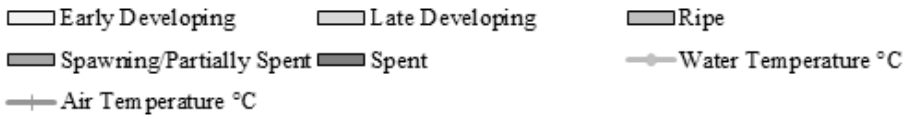


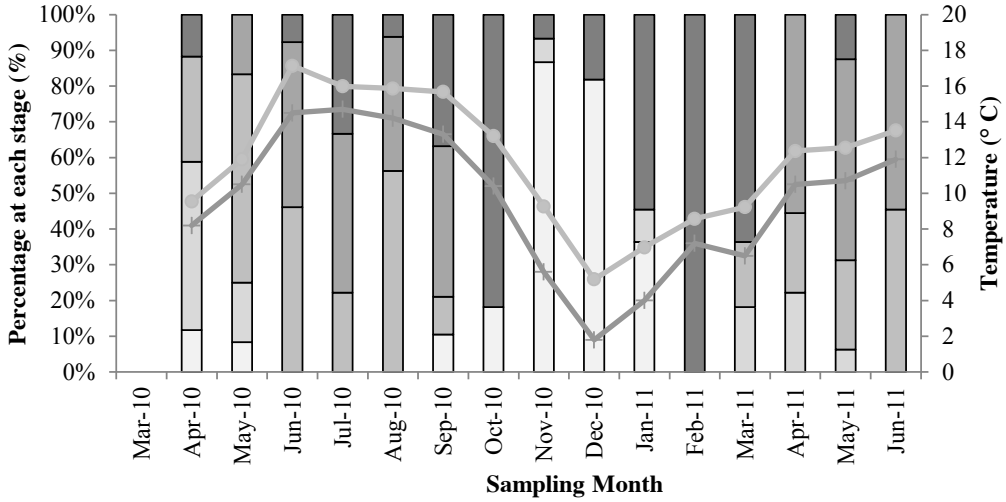
Figure 7: Gonadal development of male and female cockles at Bannow Bay.

Flaxfort Strand

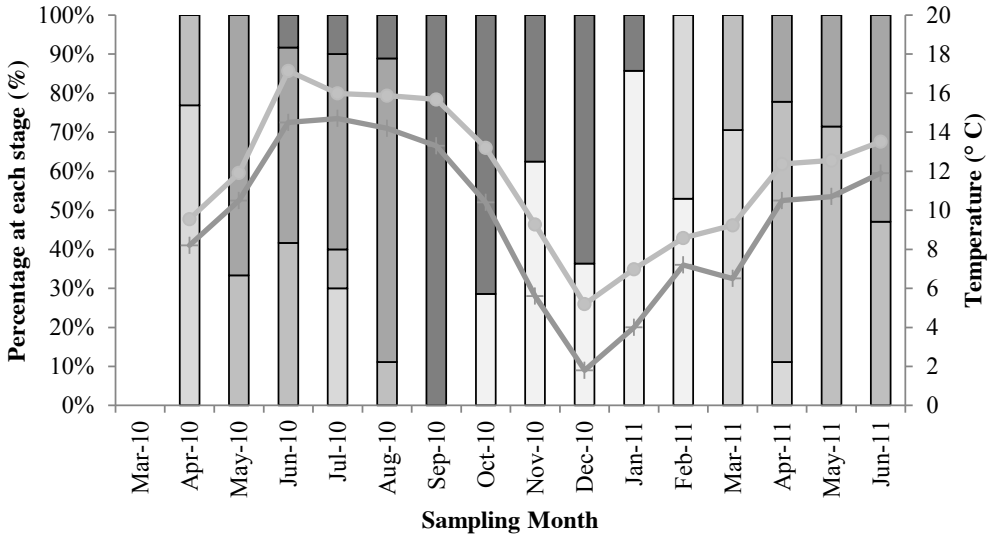
The study did not begin until April 2010 in Flaxfort Strand and the majority of males were late developing (76.9%) but some were ripe (23.1%) (Figure 8). The spawning duration lasted from May 2010 until August 2010 and by September 2010, 100% of males were spent. Gametogenesis among males was visible from October 2010, but cockles developed slowly remaining as early developing until February when the later stage of gametogenesis was seen. Male cockles became ripe (29.4%) in March 2011 and spawned from April 2011 (22.2%) spawning continued until the end of the study. However until June 2011 a greater percentage were ripe rather than spawning.

A greater range of gonadal stages were seen among females in April 2010, than in males; early developing (11.8 %), late developing (47.1%) and ripe (29.4%). Spawning began in May 2010 (16.7%) and continued until September 2010 (42.1%). Gametogenesis began in September 2010 (10.5%) while other females were still ripe (10.5%) and spawning (42.1%). Development continued until more females became ripe in March 2011 (18.2%) and began spawning a month later in April 2011 (55.6%). The peak spawning time for females in 2011 was in May 2011 (56.3%) and decreased marginally to 54.5% in June 2011. Hence spawning and its peak were a month later at Flaxfort Strand than Bannow Bay.

Male



Female



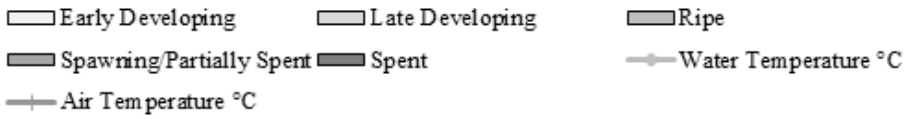
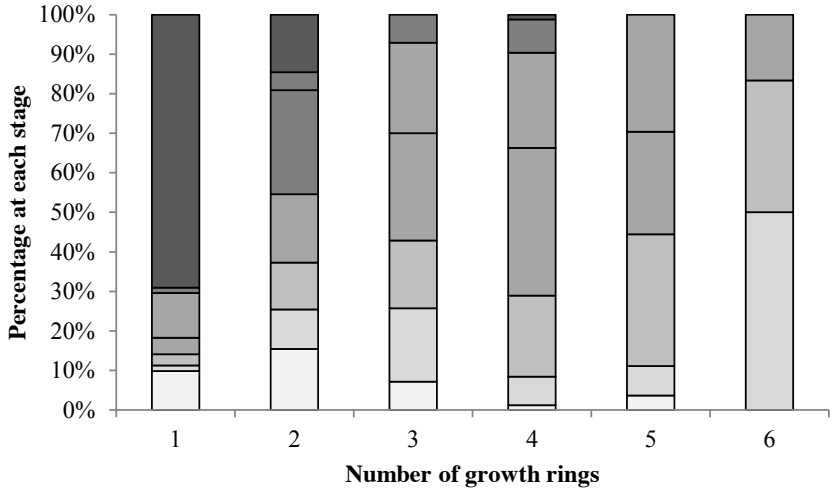


Figure 8: Gonadal development of male and female cockles from Flaxfort Strand.

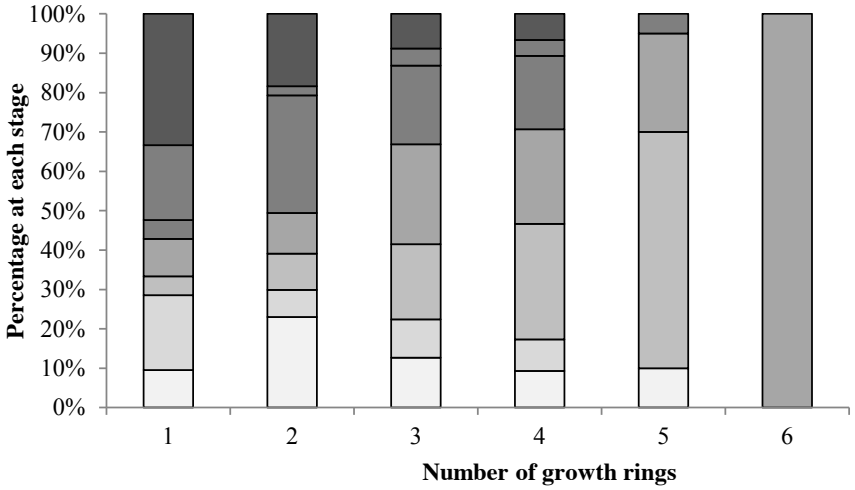
3.4.4 *Gonadal Maturity and Age*

The smallest shell length in which gonads were observed was 8.9 mm in Flaxfort Strand and 9.9 mm in Bannow Bay. Both were females in the early developing stage with one growth ring but the majority of cockles with one growth ring at both sites did not develop gonads (BB 69.0%; FF 33.3%). Other stages of development were apparent in cockles with one growth ring too, these were early developing (BB 9.9 %: FF 9.5 %), late developing (BB 1.4 %: FF 19.0 %), ripe (BB 2.8 %: FF 4.8 %), spawning/partially spent (BB 4.2 %: FF 9.5 %), spent (BB 11.3 %: FF 4.8 %) and indeterminate (BB 1.4 %: FF 19.0 %). The majority (90%) of animals did not develop gonads until they were above 11 mm in length with one growth ring. At Bannow Bay the greatest number of ripe (30.9%) and spawning (38.8%) animals had four growth rings whereas at Flaxfort Strand the majority had three growth rings (ripe 47.6%, spawning 59.1%) (Figure 9).

Bannow Bay



Flaxfort Strand



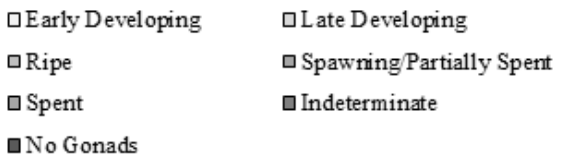


Figure 9: Percentage of each stage of gonadal maturity, indeterminate gonads and no gonads per number of growth rings.

Table 2: Size and weight data of *Cerastoderma edule* from both sites summarised.

Measurement	Bannow Bay	Flaxfort Strand
Length mm	23.2 ± 5.37	22.4 ± 2.41
Range	3.2-38.1	7.2 - 30.2
Width mm	24.7 ± 5.67	24.5 ± 2.21
Range	4.1-41.1	7.6 - 33.3
Height mm	19.8 ± 6.21	18.6 ± 1.76
Range	1.5-32.9	5 - 29.1
Whole Weight g	9.8 ± 4.87	7.4 ± 1.26
Range	0.02-34.2	0.28 - 16.5
Tissue Weight g	2.1 ± 1.02	1.5 ± 0.30
Range	0.02-6.1	0.04 - 3.1
Shell Weight	6.4 ± 3.23	4.7 ± 0.82
Range	0.02-23.8	0.12 - 10.6

3.4.5 *Gonadal maturity and temperature*

Ripe gonads were seen in April 2010 at both sites in cockles when the mean monthly temperature was 9.0 ˚C (Bannow Bay) (Figure 7) and 9.55 ˚C (Flaxfort Strand) (Figure 8). Spawning was initiated in 2010 at Bannow Bay at 15.29 ˚C (May 2010) and in Flaxfort at 11.91 ˚C (May 2010). In the following year, ripeness and spawning began at different temperatures; ripe gonads were seen in males from Bannow Bay when the temperature was 9.13 ˚C (March 2011) and the majority spawned at 15.09 ˚C (June 2011), whereas females were identified as ripe and spawned at 12.93 ˚C (April 2011). Ripening of cockle gonads in Flaxfort Strand in 2011 occurred when the mean monthly temperature was 9.23 ˚C (March 2011) and spawning at 12.37 ˚C (April 2011).

3.5 DISCUSSION

The cockle plays a significant role in soft sediments and bays where it is found in abundance and climate is one factor, mainly through temperature variation, that can impact on both reproductive capacity and recruitment and therefore overall biomass of the bivalve. Other parameters such as competition and food availability that were not measured in this study may also impact upon reproduction and growth, however long term changes in climate may alter these too. Results would indicate that climate variability is impacting on reproduction of this bivalve by prolonging the period over which spawning extends. Whether in the long term this translates into more recruitment will depend on the external factors that contribute to this process e.g. plankton blooms and how they are affected by this climate change in terms of when they occur and which particular species will dominate.

Temperatures recorded over the study period were anomalous for Ireland, the winters of 2009-2010 and 2010-2011 were exceptional for several reasons: the winter of 2009-2010 was the coldest winter since 1962-1963. Additionally it was the sunniest winter on record and had a lower frequency of strong winds. Whereas the winter of 2010-2011 was noteworthy as the cold spell began earlier in November 2010 than in previous years, it also had the coldest day measured in Ireland and nine uninterrupted days during which the temperature did not rise above 0°C (www8). Summer temperatures in 2011 were also lower than normally experienced. This period of extreme cold was in comparison to warming trends on land and sea in Europe, with the average land temperature from 2002-2011 being 1.3°C higher than pre industrial levels (www9). It is believed the cold temperatures were as a result of the negative phase of the North Atlantic Oscillation (NAO) which was at its most negative value (for which there are data) and North Atlantic atmospheric blocking conditions (Cattiaux *et al*., 2010; Sillmann *et al*., 2011; Vicente – Serrano *et al*., 2011). Whether the NAO index is positive or negative is not thought to be impacted by anthropogenic warming, however it may influence multidecadal variability, with extended time spans of extreme positive or negative NAO index possibly becoming more common if temperature increases persist (Goodkin *et al*., 2008). Temperatures recorded in 2010 were higher than the corresponding months in 2011 at both sites and there were site differences in temperature. There are differences in the tidal cycle (its duration) between the sites, which could have contributed to the differences observed in temperature.

Cockles from Bannow Bay had a higher weight than cockles from Flaxfort Strand, but a greater number of cockles at Bannow Bay had less growth rings, indicating that overall cockles at this site grew faster than at Flaxfort Strand. The difference in cockle lengths and condition index (although not significant) and weights between the two sites could be linked firstly to immersion duration providing longer deeding time, secondly the detrimental effects of prolonged emersion and oxygen debt and thirdly to the extent of macroalgal growth (de Montaudouin 1996); all of which are factors, which differ between the sites. Previous studies of cockle pathology at Flaxfort Strand have shown that the frequency of digenean trematodes and disseminated neoplasia are highest in intermediately (18.0 – 23.9 mm) sized cockles, so many cockles may die before attaining maximum size (Morgan *et al*., 2012).

There were a significantly greater number of cockles with two or less growth rings at Bannow Bay than Flaxfort Strand, which could be related to site differences in water quality as Flaxfort Strand is eutrophic and Bannow Bay is of good water quality. Under stressful conditions it has been shown that another bivalve - *Mytilus edulis* produces less viable larvae (Bayne *et al*., 1978) and bivalve larvae themselves are highly susceptible to the effects of pollutants such as heavy metals. Roads surround Flaxfort Strand, unlike Bannow Bay, and there may be run off of hydrocarbons during rains and these pollutants may affect the recruitment of cockles in this area (Beiras and His, 1985). There were higher densities of cockles at Flaxfort Strand than at Bannow Bay (preliminary study), therefore high densities of larvae could be filtered out of the water column before settlement.

More females were identified than males but no significant deviation from a 1:1 ratio was observed in agreement with previous Irish and English studies (Boyden, 1971; Morgan *et al*., 2012). A number of indeterminate cockles and one possible hermaphrodite were seen at Flaxfort Strand. Cockles of all size classes were collected, leading to the gathering of sexually immature cockles; additionally some cockles' gonads upon examination were castrated by trematodes (personal observation). 22% of cockles in a study from the Crouch Estuary, England were of indeterminate sex (Boyden, 1971). One hermaphrodite was also found in the study on the Kent coast, but this was a mixed study of *Cerastoderma edule* and *Cerastoderma glaucum* and the species was not stated.

Gonads were observed in cockles with one growth ring. Gametogenesis in cockles has previously been recorded in animals with one growth ring (Cardoso *et al*., 2009). However

this precocious development was rare, with most not developing gonads until >11 mm. In another study of cockle reproduction in the Dutch Wadden Sea, gonads were identified in cockles > 12 mm (Cardoso *et al*., 2009). At Strangford Lough, initial development of gonads was recorded in cockles from 15-20 mm (Seed and Brown, 1977). According to Dabouineau and Ponsero, (2009) size, not age of cockles, could be of greater importance to cockle sexual maturity. The majority of spawning cockles had three or four growth rings in the present study. This is in contrast to cockles from the Burry Inlet, Wales where precocious spawning occurred with the majority of cockles with one growth ring spawning and subsequently dying, perhaps as a reaction to high densities (Elliot *et al*., 2012). One or two year classes disproportionately contributing to the overall cockle population has been described previously in the Wadden Sea (Cardoso *et al*., 2009) and greater production by older cockles has also been described in the Mundaca Estuary, Spain (Iglesias and Navarro, 1991).

In the present study there were differences observed between the sites and between the sexes in peaks of gonadal maturity, spawning duration and period. Two peaks in spawning were observed at both sites, which were also previously recorded in Ireland (Morgan *et al*., 2012). Cockles were ripe in both sites from April in 2010, but March in 2011. Advancement of gonadal maturity was seen in male soft shell clams, *Mya arenaria* to March and April 2011 in comparison to 2010 when most males developed in May, at Bannow Bay that was studied over the same time period (Cross *et al*., 2012). Ripe gonads have been described previously in cockles from May in the south east of England (Kingston, 1974) and in another study in Strangford Lough; males were described as ripe from April but females not until May (Seed and Brown, 1977). This study may be demonstrating that previous severe winters may be accelerating gametogenesis with ripe individuals being observed earlier in the year. Yankson (1986) measuring fecundity with gamete volume fraction (GVF), reported an earlier peak in fecundity among cockles in the Burry Inlet in 1982 after a severe winter compared to the previous year.

The spawning period extended until November in Bannow Bay, but ceased in September in Flaxfort Strand. The spawning period in intertidal cockles in the Dutch Wadden Sea continued until October (Cardoso *et al*., 2009), but in an earlier study in Ireland the majority of cockles were in a spent condition from August/September (Seed and Brown, 1977). Differences in spawning period could be due to site conditions, cockles at Bannow Bay were

heavier than at Flaxfort Strand, and there may be a greater availability of nutrients to the cockles for gonad production in Bannow Bay (Cotter *et al*., 2010). If there is a greater amount of gonads it may take a longer time period to spawn. It has been suggested also that reproductive strategy of a species may not be set but could vary, with the prevailing conditions influencing the strategy adopted (Yankson, 1986).

Partial spawning was observed in this present study, with a low number of individuals remained partially spent over winter with residual gametes apparent; this was particularly evident at Bannow Bay. Complete spawning was found among *Cerastoderma edule* in the Crouch Estuary and the Dutch Wadden Sea (Boyden, 1971; Cardoso *et al*., 2009). Gametogenesis was seen in both sites over winter but at both sites the sexes were not in synchrony. Navarro *et al*. (1989) proposed that gametogenesis begins again over winter if sufficient glycogen reserves have been amassed during the previous summer. Rapid gonadal development initiated in spring at Strangford Lough, Northern Ireland, has been described at the beginning of the growing season after over wintering in spent condition (Seed and Brown, 1977; Cardoso *et al*., 2009) however slow gonad development has been described in the Crouch Estuary, Essex and in the Burry Inlet (Boyden, 1971; Yankson, 1986). In the latter scenario growth begins in autumn and continues over winter with increased impetus in spring and spawning commences in late spring/early summer (Boyden, 1971; Yankson, 1986; Twomey, 1987).

It has been stated, that in cockles, temperature does not have a coordinating effect on reproduction, either by a minimum threshold required or by temperature change (Navarro *et al*., 1989). However changes in the condition index of cockles from St-Pol-de-Léon, France, from 1987 to 1989, reflected alterations in temperature, with lows during the colder year of 1987 in comparison to highest condition index during 1989 which was warmer than average (Guillou and Tartu, 1992). Other studies indicate increases in temperature, not its absolute value, influence cockle reproduction (Dabouineau and Ponsero, 2009). In the present study ripening and spawning of cockles at both sites occurred at different temperatures between years as was also observed in the Mundaca Estuary, North Spain (Navarro *et al*., 1989) and prevailing environmental conditions altering gametogenesis and physiology in cockles has been suggested (Iglesias and Navarro, 1991). Gonadal maturity could be attributed to not only temperature but also the animal's condition and food availability (Yan *et al*., 2010).

This study coincided with two severe winters, anomalous to Ireland (www 5). Particularly cold winters may additionally aid reproductive success in cockles by ensuring that male and female gametes are released in unison (Dabouineau and Ponsero, 2009). A study of cockle gametogenesis in the Burry Inlet in 1981-1982 corresponded as well with an unusually cold winter. Their findings (Yankson, 1986) included incomplete spawning, followed by redevelopment and spawning again, which was in agreement with the incomplete spawning seen in the present study.

Milder winters experienced in the Wadden Sea area have been linked to on-going reductions in bivalve recruitment (Philippart *et al*., 2003), whereas several studies have reported greater bivalve recruitment after severe winters (Honkoop *et al*., 1998; Philippart *et al*., 2003; Beukema and Dekker, 2005). Less severe milder winters are thought to allow for gametogenesis to occur for longer, therefore not allowing for a cessation in gonad production and a rest period for the bivalve, but colder winters provide a rest period between cycles which may be advantageous for redevelopment in spring (Guillou and Tartu, 1992). Levels of interannual variation can be observed nonetheless in many marine invertebrates and this phenomenon is not totally understood (Honkoop *et al*., 1998). Direct (variation in egg numbers) and indirect (predator-prey mismatch) effects of climate change are thought to be responsible for this occurrence (Flach, 2003). Cardoso *et al*. (2009) believe that *C*. *edule* may be more affected by climate change than other more long-lived bivalves such as *Mya arenaria* as the possibility of them experiencing a cold winter during their short lifespan is low.

Moore *et al*. (2011) postulate that comparing historical and contemporary data has limitations but may be informative in relation to species responses to climate change in particular, if baseline data are absent. Although different methods of assessment were used, it appears that the spawning period of cockles has extended throughout the last century (Table 3), with an earlier starting time and continuing later into autumn. This could be linked to the change in environmental conditions, orchestrated by global climate change, with globally oceans warming on average by 0.6 °C in the last 100 years (Wiltshire *et al*., 2008) and European seas are thought to be especially affected by climate change (Philippart *et al*., 2011). Alterations in phenological relationships due to sea temperature will have consequences modifying many trophic interactions such as food webs (Edwards and Richardson, 2004; Wiltshire *et al*.,

2008). Indeed marine temperate areas could be especially susceptible to changes in the cycle of plankton production as recruitment is contingent upon it and spring plankton blooms are occurring earlier (Edwards and Richardson, 2004; Heath *et al*., 2012). Therefore earlier spawning in cockles and longer spawning period, could be as a consequence of amendments to plankton pulses. Extension of the breeding season could reduce the chances of a mismatch with peaks in primary production and prevent the chances of overpopulation by mass spawning, as has been described in *Cerastoderma glaucum* (Guillou and Tartu, 1992). This could be exacerbated in cockles, since as previously stated, as income breeders, they can quickly utilise food resources for gonad manufacture (Cardoso *et al*., 2009). Other invertebrates such as the brown shrimp *Crangon crangon* have altered their spawning regime in response to warmer temperatures by having two spawning periods in the southern North Sea, compared to one in the northern North Sea (Heath *et al*., 2012). Similarly the boreal limpet *Patella vulgata* spawns further into the autumn in the 2000s than it did during the 1940s (Moore *et al*., 2011).

Table 3: Previous study locations and methods of assessing *Cerastoderma edule* gametogenesis arranged by latitude.

Location	Method	Gametogenesis	Spawning Period	Reference
Trondheim, Norway	Gonad smears		Early July began	Rygg, 1970
Western Wadden Sea	Gonadal Mass Ratio		April - October	Cardoso *et al*., 2009
Strangford Lough, Ireland	Gonad classification	Spring	Midsummer - Aug	Seed and Brown, 1977
Cardigan Bay, Wales	Histology	October - March	May began	Creek, 1960
Crouch Estuary, England	Gonadal smears	Early Spring	May - July	Boyden, 1971
Burry Inlet, Wales	Gonad smears		June began	Hancock and Franklin, 1972
Kent Coast, England	Histology	September and October	May and June	Kingston, 1974
Plymouth, England	Sieving		May - August	Lebour, 1938
Mundaca Estuay, Basque Country, Spain	Gamete volume fraction	November	May - September	Navarro *et al*., 1989

Table 4: Summary of main results.

Temperature:	
	No significant difference between sites, but 2010 was warmer than the corresponding months in 2011.
General Cockle Biology:	
	Cockles from Bannow Bay greater in length, width, and shell height (but not significantly so).
	Significantly greater whole weight, shell weight, and tissue weight in Bannow Bay.
	Greater condition index at Bannow Bay (but not significantly so).
	Sex ratio adhered to 1:1, but females were more common at both sites.
	Significantly more young cockles (with less than two growth rings) at Bannow Bay
Reproduction:	
	Five stages of gonadal maturity identified.
	After a severe winter gonad ripening and spawning began earlier.
	Ripening and spawning were recorded at different temperatures.
	The smallest cockle with gonads was 8.9 mm (Flaxfort Strand) and 9.9 mm (Bannow Bay).
	Most cockles (90 %) did not develop gonads until they were above 11 mm in length.
	Cockle gametogenesis appears to have extended in duration, especially when compared to other studies.
	Gametogenesis began over winter, in contrast to previous study in Ireland.

In spite of trends in the Wadden Sea and western Sweden towards milder winters, this present study was conducted during a time of uncharacteristically cold winters in Ireland, which appears to bode well for cockle recruitment success. However, this was followed by a mild winter in 2011-2012, so it is difficult to predict future winter conditions. Although previously in Ireland gametogenesis has been described from spring only, this study found that cockles begin to develop gonads over winter. The duration of spawning time reported in this study also contrasts with previous studies, starting earlier and continuing until later in autumn. Males and females in both sites additionally, were not entirely synchronous in terms of gametogenesis and spawning, which if the timespan widens could impact negatively upon spawning success (for a summary of the main results refer to Table 4). The lack of younger year classes of cockles present at Flaxfort Strand could have serious ramifications for the many birds that rely upon them as a food source. Particularly birds classified as Annex 1 on the EU Birds Directive e.g. bar tailed godwits and golden plovers frequent this area.

Alterations in cockle gametogenesis, spawning and subsequent settlement are not only dependent on temperature but also the effect that climate variability has on plankton and previously appropriate habitats may no longer be suitable due to changes in oxygen levels or thermal regime. Reductions in cockle numbers would have concurrent effects on water chemistry, sediment oxygenation and structure and the species richness of an area. Further studies using other intertidal organisms such as mussels or barnacles could be beneficial, to see if changes in spawning, or larval release timing is widespread in marine shallow water invertebrates.

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CHAPTER 4: REPRODUCTION IN SOFT SEDIMENT BIVALVES

SECTION 1: Plasticity in bivalve reproduction: identifying drivers of variation in *Mytilus edulis*.

4.1 ABSTRACT

Reproduction and gametogenesis in marine invertebrates, in general, and in the mussel in particular are in part driven by exogenous factors such as food availability and environmental factors such as temperature, and are modulated by stressors such as extremes in these factors and the presence of pathogens and disease. The rapidly changing marine environment and alterations in climate may have unprecedented impacts on gametogenesis and recruitment in the mussel and other invertebrates and, in turn, on their demographics and abundance. This may have far reaching consequences, as not only are mussels a major shellfish commercially for food, but they play a vital role in the environment also. Reproduction of the mussel was studied at two sites in Ireland, 215 km apart and of contrasting water quality, over a 16 month period. Though general climate change predictions for Ireland indicate a warming trend, the study period included an anomalous severe winter, the coldest in 50 years. The study indicated that populations can use different reproductive strategies as was reflected in the variation in size of mature animals and the number of spawning periods. Gametogenesis began earlier in this study than has been previously reported, especially after the exceptionally harsh winter, where alterations in the reproductive cycle were apparent. A smaller range of size classes spawned at one site relative to the other. Males and females were synchronised after the cold spell, and there was advancement and extension of the spawning period, indicating that such events may be of benefit to this bivalve. This rapid response to exogenous factors demonstrates the plasticity of this species of bivalve and its ability to take advantage of prevailing conditions.

Keywords: *Mytilus edulis*, climate variability, gametogenesis, nutrition, growth rates.

4.2 INTRODUCTION

Approximately US $ 14 trillion worth of ecosystems goods and services are provided by the neritic marine ecosystem annually (Harley *et al*., 2006). This figure includes

food, nutrient cycling and disturbance regulation. A key exploited species is the blue mussel, *Mytilus edulis*. Exploited for food, bait and fertiliser since 6000 B.C. (www1) *M. edulis* is undoubtedly one of the most commonly recognised marine bivalves present on the shore and in the shellfish/food trade to this day. *Mytilus edulis* accounts for 8% of world mussel production by species – and its role in aquaculture has introduced it into China, Namibia and the French Mediterranean coast (www1; Beaumont *et al*., 2007). Restrictions on collection of seed due to environmental laws have been imposed, which may be difficult for aquaculture as it mostly relies on a wild seed supply (www1; www2; de Vooys, 1999; Beaumont *et al*., 2007; Domínguez *et al*., 2010).

In addition to its commercial and economic importance, *Mytilus edulis* plays a vital role in its environment. In the Dutch Wadden Sea, 23% of the intertidal biomass can be mussels, this can increase up to 67% in the subtidal region (Dankers and Zuidema, 1995; de Vooys, 1999). *Mytilus edulis* can form large biogenic reefs due to its often high densities in its habitat; which provides a substratum for barnacles and macroalgae in addition to creating habitat heterogeneity and refuges for smaller invertebrates such as crabs, gastropods and polychaetes (Dankers and Zuidema, 1995; Anonymous, 2010). Many bird species are dependent on mussels as a food source, in the late 1980s and early 1990s high mortalities of oystercatchers and eiderducks was related to low mussel biomass (de Vooys, 1999; Berge *et al*., 2006, Anonymous, 2010). *Mytilus edulis* accelerates the breakdown of organic matter, produces ammonia and silicates and deposits suspended matter as faeces and pseudofaeces (Dankers and Zuidema, 1995).

Mytilus edulis, being holarctic (Secor *et al*., 2001), is widely distributed from the Russian White Sea to the Atlantic coast of southern France, with further pockets from Canada to North Carolina, Chile, Argentina, the Falkland Isles and the Kerguelen Isles (www1; Newell *et al*., 1982; McKenzie, 1986; Tyler-Walters, 2002; Berge *et al*., 2006). Its wide distribution is due to its eurythermal status (it can tolerate freezing conditions; its temperature range is typically 5 – 20 °C with an upper sustained thermal limit of 29°C) and its ability to withstand desiccation and low oxygen conditions (www1). Furthermore it is euryhaline tolerating a range of

salinities from 4 to fully oceanic, but its growth rate is reduced below 18, and does not flourish below 15 (www1; Sunila, 1981) and as such it is commonly encountered from the intertidal to subtidal.

Mussels reach sexual maturity in their first year (Seed and Brown, 1977; Sunila, 1981; McKenzie, 1986) and sexual maturity is a function of age not size, as growth rates can vary considerably between localities dependent on environmental conditions (Seed, 1969). They are capable of reproducing until death (in the wild they can live 18 – 24 years (www1; McKenzie, 1986). Their reproduction is referred to as "conservative", as large energy reserves that they accumulate can shield them from the potential effects of environmental change (Newell *et al*., 1982; Hawkins *et al*., 1985). They are gonochoristic with no evidence of sexual dimorphism but hermaphrodites do occur (Seed, 1969; Sprung, 1984; McKenzie, 1986; Beaumont *et al*., 2007). Up to 6% of *M. edulis* total tissue weight may be comprised of gametes but after spawning this is reduced to zero (Secor *et al*., 2001; Beaumont *et al*., 2007; Domínguez *et al*., 2010). In European waters, gonads become ripe in spring and again in summer, following spawning. Release of gametes is orchestrated to maximise the chances of successful reproduction, coinciding with increased food supplies for both larvae and adults. This has a dual advantage, it is beneficial for new recruits and it allows for energy acquisition prior to the next reproductive event for adults (Newell *et al*., 1982). Fertilisation is external (Bayne *et al*., 1983) and embryos develop into veliger larvae which become members of the meroplankton 48 hours after fertilisation. They persist in the plankton for 4 – 8 weeks, before primary settlement occurs once their shell length reaches 250 – 300 microns (Beaumont *et al*., 2007). Secondary settlement may occur via byssal drifting (Beaumont *et al*., 2007).

Exogenous factors, such as temperature and food, are important to the timing of gametogenesis and spawning, however variation may be apparent in terms of commencement and duration at different latitudes plus interannual variation has been reported (www1; Newell *et al*., 1982; Snodden and Roberts, 1997; Domínguez *et al*., 2010). The complex mechanisms by which these exogenous factors act in combination with endogenous ones, like nutrient reserves and genotype, are

unknown (Seed 1969; Newell *et al*., 1982). Trends toward greater recruitment following cold winters are apparent though, linked primarily to predator prey mismatch (Strasser, 2002; Anonymous, 2010) but not to a physiological response such as a greater mass of gametes (Strasser and Günther, 2001).

The effects of global climate change are quite apparent in the North Atlantic and the Irish Sea area; 1965 – 2004 has been characterised by net heat accumulation in the North Atlantic and the sea surface temperature around the UK and Ireland has increased at six times the rate of the global average (Frost *et al*., 2012). From 1870 – 2007 in the Irish Sea surface temperatures have warmed by 0.5 to 1 °C (UKMMAS 2010). For the preceding 30 years there have been increases of 0.2 – 0.6 °C in sea temperature per decade around the UK and Ireland. The mean surface ocean pH has reached 8.14 in comparison to 8.25 in the 18th century (Heath *et al*., 2012). Since pre-industrial times, it is estimated that there has been a decrease of 0.1 pH units until now in sea surface waters and a further decrease of 0.4 pH units is expected by the end of the 21st century (Berge *et al*., 2006). The northern hemisphere has been warmer since 1980 than at any time in the last 2000 years and the mean annual air temperature recorded in Ireland has increased by 0.7°C in the period 1890 – 2004 (Philippart *et al*., 2011). However the present study was performed at a time when there were two unusually cold winters in Ireland, which proved to be the coldest in 50 years (Met Eireann).

Although much is made of the commercial importance of mussels, they are also an integral part of the marine ecosystem, acting as habitat, a food source, and similarly alter water chemistry and nutrient availability. Although mussels are known for their plasticity and ability to exploit many contrasting habitats, the current abiotic variations in the marine environment are rapid and may be happening too swiftly for organisms to buffer them. Populations of *Mytilus edulis* may differ greatly in terms of growth and reproduction (McKenzie, 1986) therefore two sites, 215 km apart, were chosen in this study of mussel population structure and gametogenesis.

4.3 RESULTS

In total 874 mussels, were screened using histology during the course of the study (Bannow Bay n = 434: Flaxfort Strand n = 440). 94.0% of mussels from Bannow Bay showed some gonadal development, whereas 90.5% of the mussels from Flaxfort Strand showed gonadal development.

4.3.1 *Temperature*

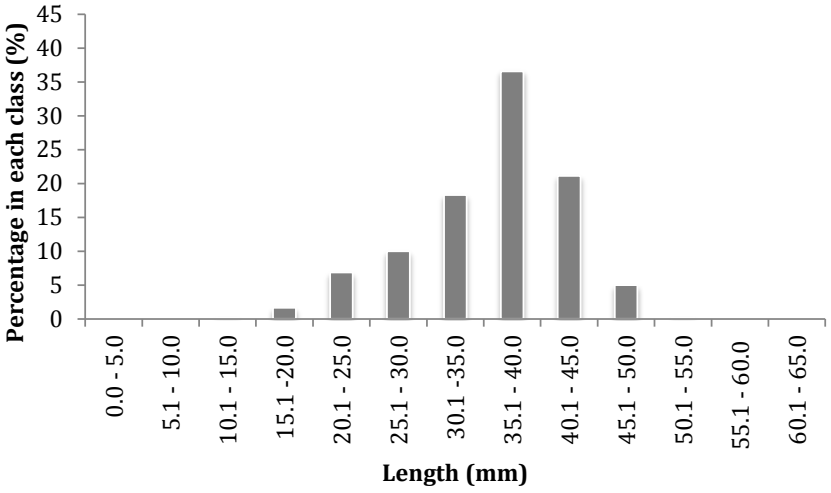
There was no significant difference in the mean water or air temperatures between the sites when temperatures from 2010 and 2011 were combined (Table 2) (Mann Whitney test). The mean air and water temperatures were higher in Bannow Bay than Flaxfort Strand but not significantly so. Both air and water temperatures showed seasonality with lows in December but highs in June. Water temperature in Bannow Bay ranged from -1.66 °C in December 2010 to 27.97 °C in June 2010 and from -0.44 °C in January 2011 to 23.36 °C in June 2011. This was in comparison to -3.36 °C in December 2010 to 26.16 °C in June 2010 at Flaxfort Strand, and -1.78 °C in January 2011 to 27.44 °C in June 2011. At both sites there were higher and lower temperatures in 2010 than in 2011.

Table 2: Summary of mean monthly temperature data at both sites: water temperatures collected using TidBit temperature loggers and addtional air temperature provided by Met Eireann.

	Bannow Bay		**Flaxfort Strand**	
	Water Temperature °C	Air Temperature °C	Water Temperature °C	Air Temperature °C
2010				
Maximum	18.0 (June)	16.5 (July)	17.1 (June)	14.7 (July)
Minimum	3.8 (December)	2.2 (December)	5.2 (December)	1.8 (December)
Mean ± s.d	13.6 ± 5.08	10.5 ± 4.66	12.6 ± 4.00	10.4 ± 4.47
2011				
Maximum	15.1 (June)	12.6 (June)	13.5 (June)	11.9 (June)
Minimum	5.7 (January)	4.7 (January)	7.0 (January)	4.0 (January)
Mean ± s.d.	10.7 ± 3.64	9.0 ± 3.12	10.5 ± 2.62	8.5 ± 3.04
Overall				
Maximum	18.0 (June 2010)	16.5 (July 2010)	17.1 (June 2010)	14.7 (July 2010)
Minimum	3.8 (December 2010)	2.2 (December 2010)	5.2 (December 2010)	1.8 (December 2010)
Mean ± s.d.	12.3 ± 4.59	10.0 ± 4.12	11.8 ± 3.57	9.6 ± 3.95

4.3.2 *General Mussel Biology*

a.



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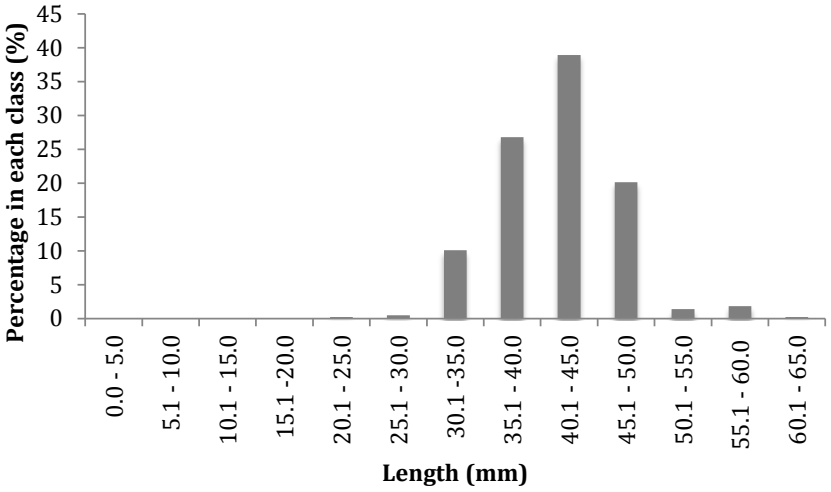


Figure 2: Percentage of mussels per length class at a. Bannow Bay and b. Flaxfort Strand (mm).

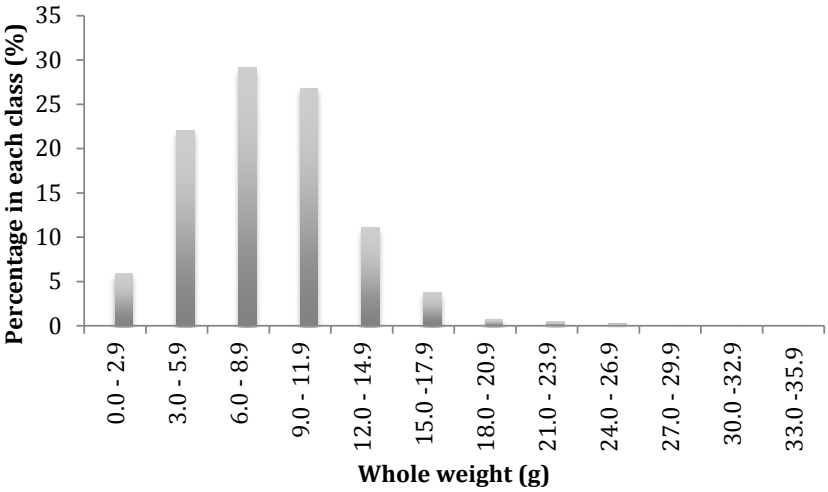
Mussels collected from Bannow Bay were significantly smaller than those collected from Flaxfort Strand in all parameters measured (Independent Samples t-test) (Table 3). Mussels collected from Bannow Bay were most frequently in the 35.1 – 40.0

mm length class (n = 154). In contrast mussels from Flaxfort Strand were most commonly of length 40.1 – 45.0 mm (n = 170) (Figure 2). There was a greater number of mussels less than 30.1 mm in size at Bannow Bay (n =79) than at Flaxfort Strand (n = 3). Most mussels from Bannow Bay had a whole weight of 6.00 – 8.99 g (n = 124). This was in comparison with Flaxfort Strand where there was a greater number of heavier mussels 9.00 – 11.99 g (n = 133). There was a greater distribution of mussel whole weights in Flaxfort Strand than in Bannow Bay (Figure 3).

Table 3: Summary of mussel measurement data of *Mytilus edulis* from Bannow Bay and Flaxfort Strand.

	Bannow Bay			**Flaxfort Strand**		
	Maximum	Minimum	Mean ± s.d.	Maximum	Minimum	Mean ± s.d.
Length (mm)	51.1	2.22	35.6 ± 5.57	60.7	13.2	41.2 ± 3.21
Height (mm)	29.8	8.6	18.9 ± 2.55	31.2	10.5	21.2 ± 2.05
Width (mm)	46.8	6.2	16.3 ± 1.75	30.1	9.6	18.4 ± 1.63
Whole Weight (g)	24.6	0.7	8.5 ± 2.16	35.7	1.2	11.7 ± 2.79
Shell Weight (g)	5.2	0.2	2.0 ± 0.48	8	0.8	2.6 ± 0.64
Tissue Weight (g)	14.9	0.4	5.0 ± 1.36	21.5	0.7	7.0 ± 1.72

a.



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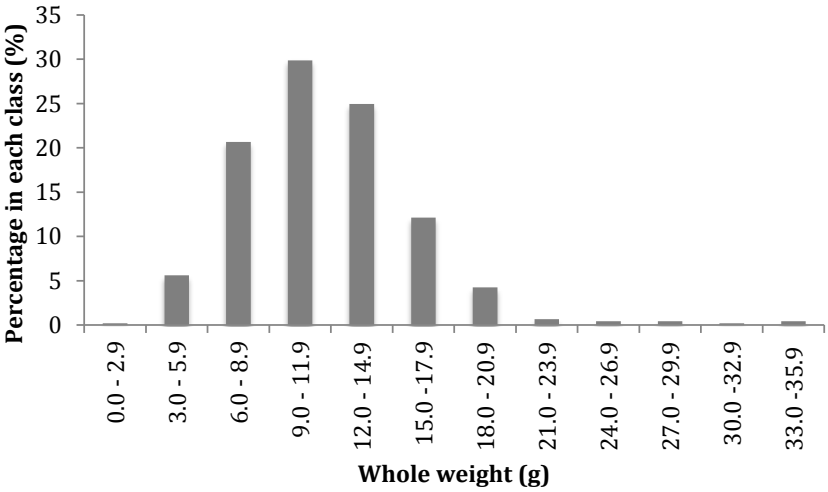


Figure 3: Percentage of individuals per weight class (g) at a. Bannow Bay and b. Flaxfort Strand.

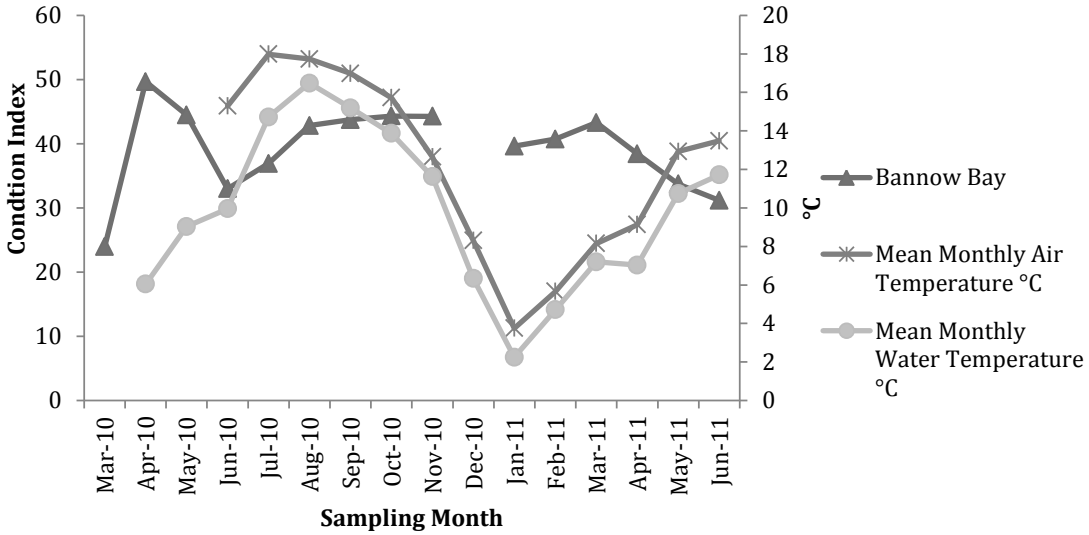
4.3.3 *Condition index*

Although there was no significant difference between the mean condition indices of mussels from Bannow Bay in comparison to Flaxfort Strand (Figure 4), the mean condition index over the study period was greater at Bannow Bay (39.3 ± 6.60) than at Flaxfort Strand (36.8 ± 4.76) even though mussels were smaller at Bannow Bay. There were also a greater number of fluctuations in the CI at Flaxfort Strand than at Bannow Bay. Furthermore, there was no correlation between condition index and temperature at either site (Pearson moment correlation).

4.3.4 *Sex Ratio and Gametogenesis*

The sex ratio at both sites was 1.05:1 with a greater number of males, but it did not deviate significantly from a 1:1 ratio (χ^2). There was a greater number of mussels of indeterminate sex at Bannow Bay (n = 89) than Flaxfort Strand (n = 54) (Figure 5).

a.



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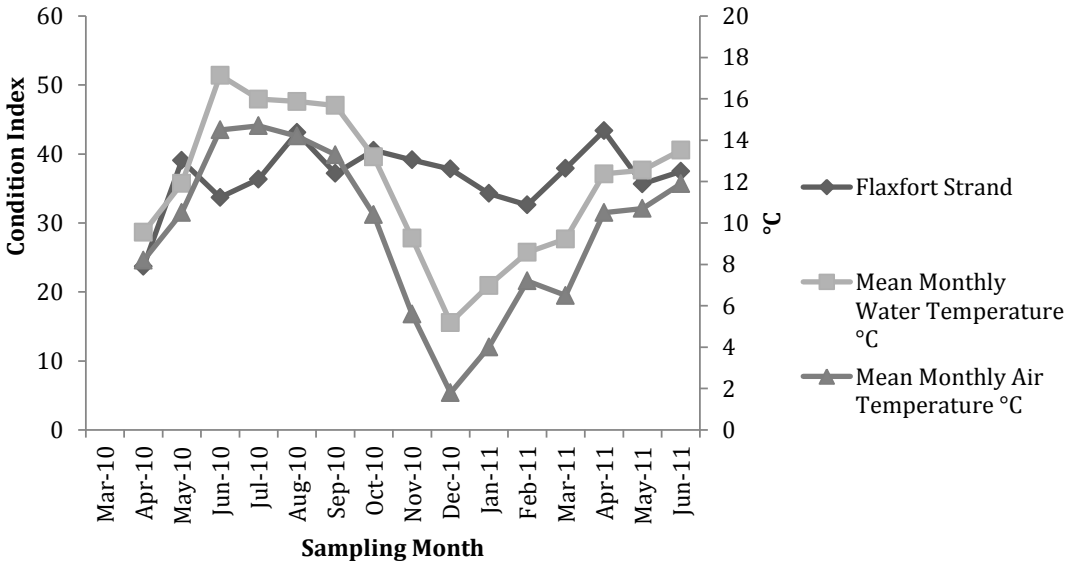
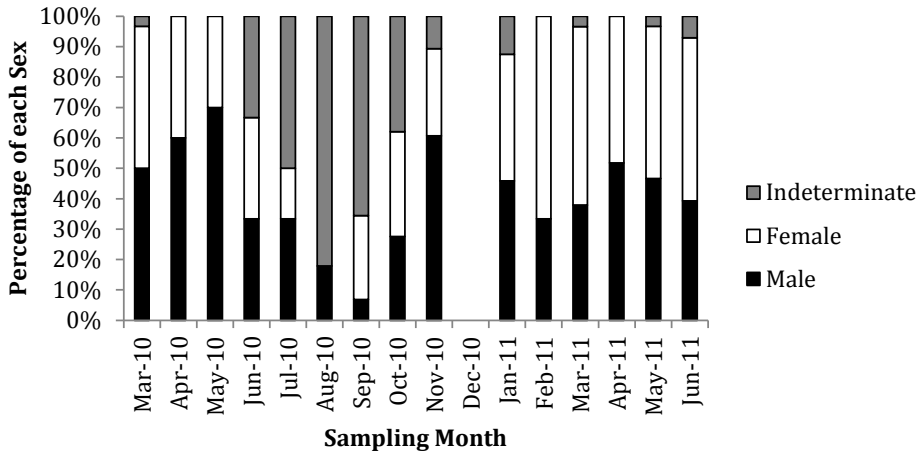


Figure 4: Condition index of mussels collected from a. Bannow Bay and b. Flaxfort Strand, with mean monthly air and water temperature included.

a.



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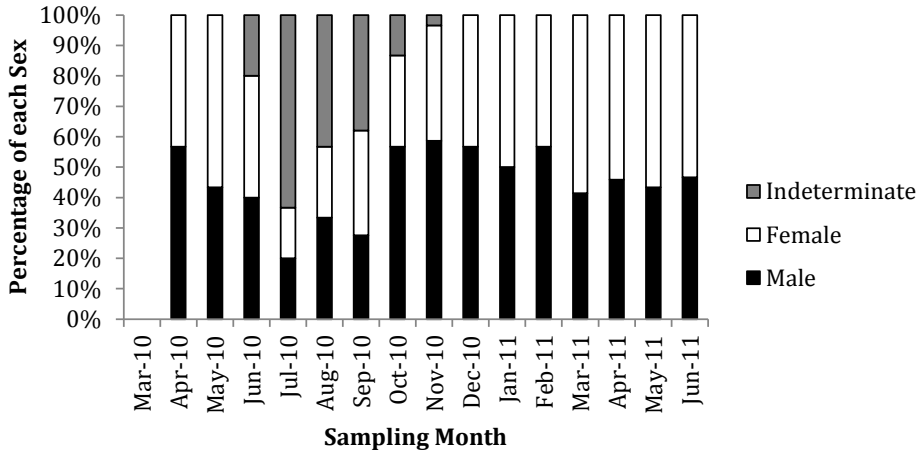
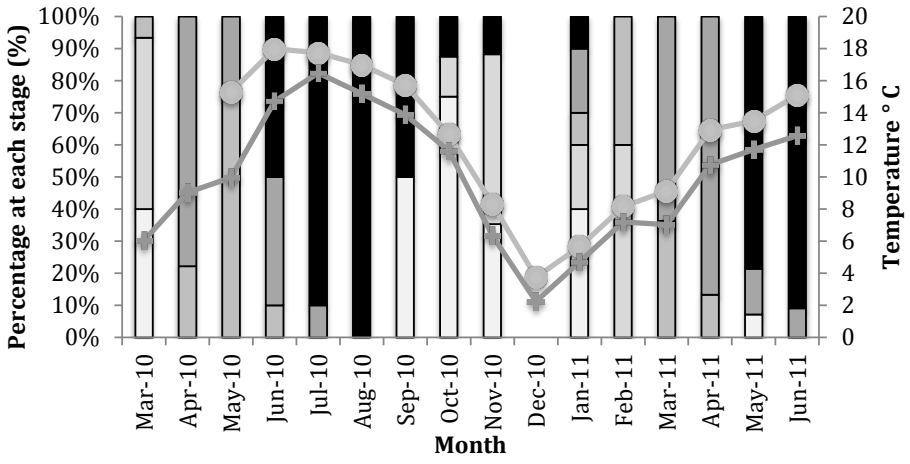


Figure 5: Sex ratio of mussels collected from a. Bannow Bay and b. Flaxfort Strand for the duration of the study.

Five stages of gonadal maturity were identified; early developing, late developing; ripe, spawning/partially spent and spent/resorbing (Table 1a and 1b). There was a maximum of five stages present in males in any one month but only three in females. Undifferentiated or totally resorbed mussels could also be seen, when it was not possible to identify sex.

In Bannow Bay ripe males were evident from March 2010 (6.7 %) until June 2010 (10 %) with a peak in May 2010 (76.2 %). A peak in spawning occurred in April 2010 (77.8 %) and continued until July 2010 (10 %). Gametogenesis began again in September 2010 when 50 % of males were early developing. Males were ripe again from January 2011 (10 %) until April (13.3 %) with spawning occurring in males from January (20 %) until June (90.9 %) when the study ended. In 2010 females were ripe from March (7.1 %) until June (20 %), with the majority ripe in April 2010 (66.7 %). Spawning occurred in June (20 %) and July 2010 (40 %). Male and female early development commenced from September 2010 (50 %). Ripe females were again identified from February 2011 (26.3 %) and spawning began in March (6.3 %) and continued until the end of the study in June (26.7 %).

a.



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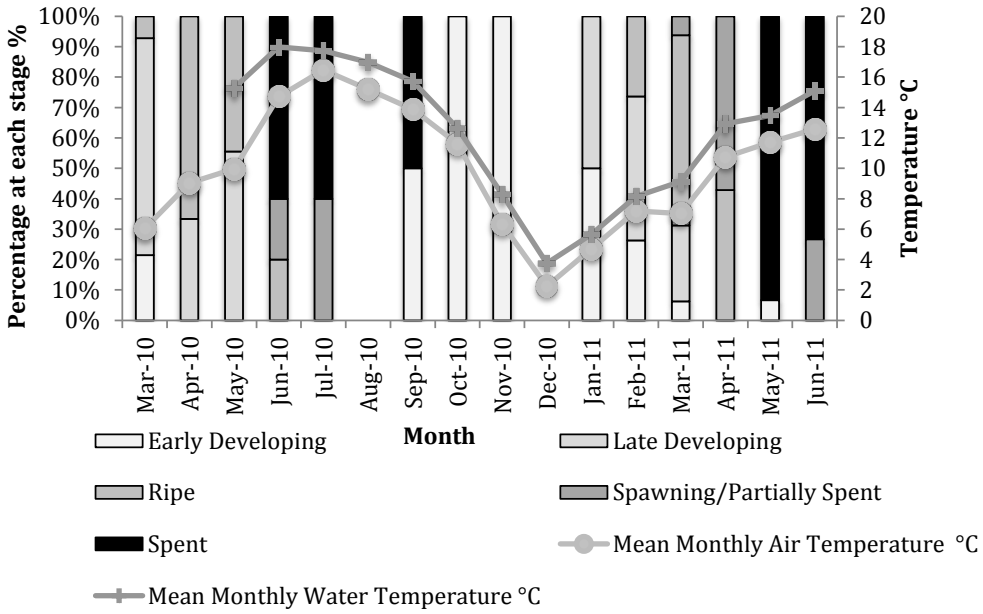
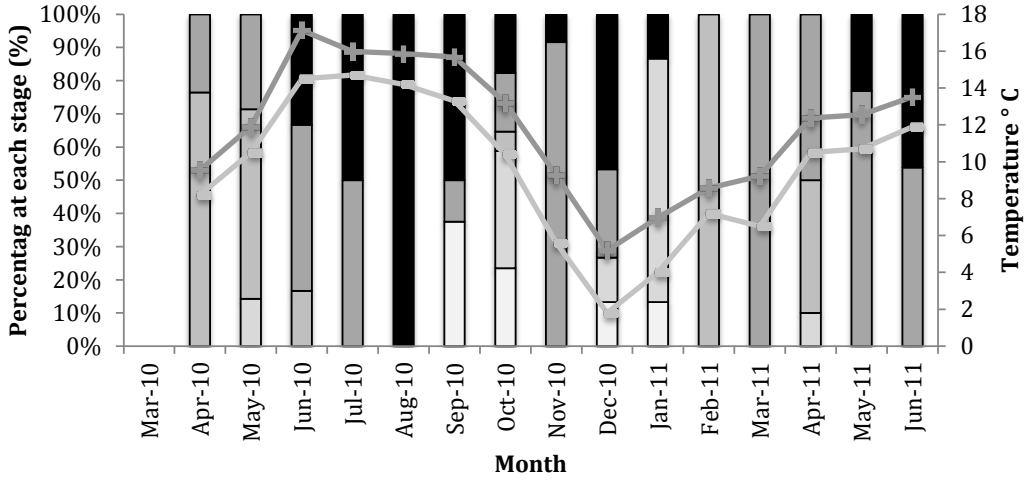


Figure 6: Gonadal maturity of a. male and b. female mussels from Bannow Bay collected throughout the study.

Upon initiation of the study in April 2010 in Flaxfort Strand, males were already in ripe condition (76.5 %) which persisted until June 2010 (16.7 %). Spawning commenced in April 2010 (23.5 %) until July 2010 (50%). Spent males were

identified from June 2010 (33.3 %) until January 2011 (13.3 %). Commencement of gametogenesis began again in September 2010 (37.5 %), the second spawning period spanned September 2010 (12.5 %) to December (26.7 %). 100 % of males were ripe in February 2011 and this decreased to 40 % by April 2011. Spawning in 2011 was at its peak in March (100 %) and was ongoing until the end of the study in June (53.8 %). Ripe gonads in females were identified from April 2010 (61.5 %) with spawning occurring in June (33.3 %) and July (37.5 %). Late developing females were recorded from October 2010 (12.5 %) up to February 2011 (23.1 %) and spawning occurred again from November (44.4 %) to December (7.7 %). Ripe gonads were clear from February 2011 (76.9 %) until May (7.1 %) with spawning occurring from March (56.3 %) until the end of the study in June (62.5 %).

a.



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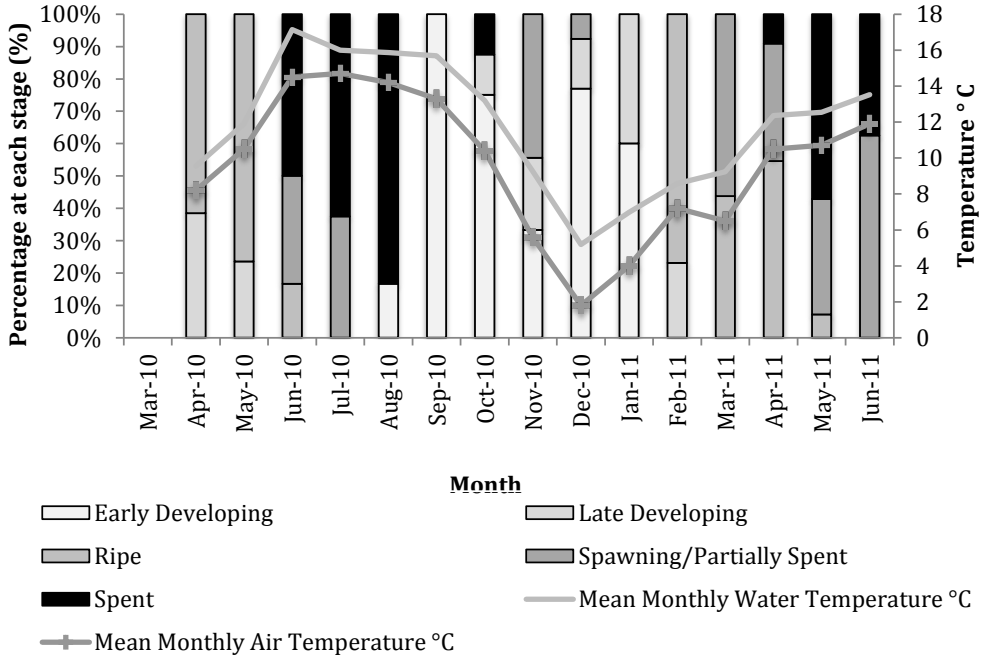
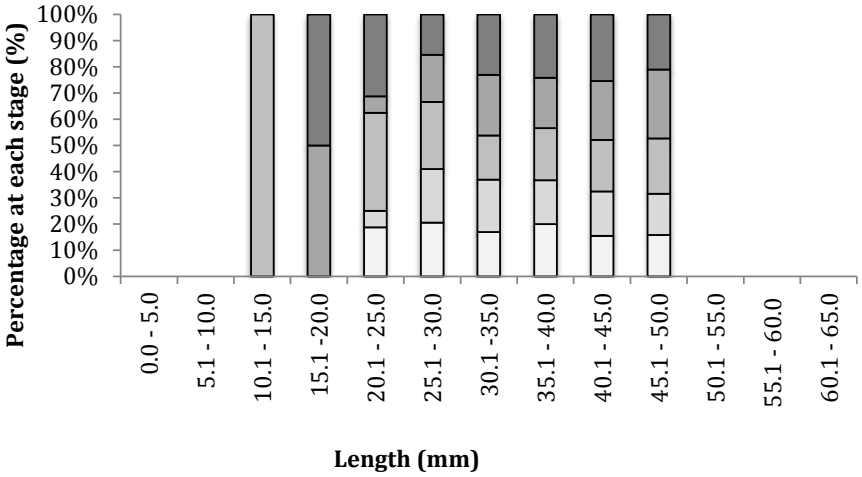


Figure 7: Gonadal maturity of a. male and b. female mussels from Flaxfort Strand for the duration of the study.

a.



b.

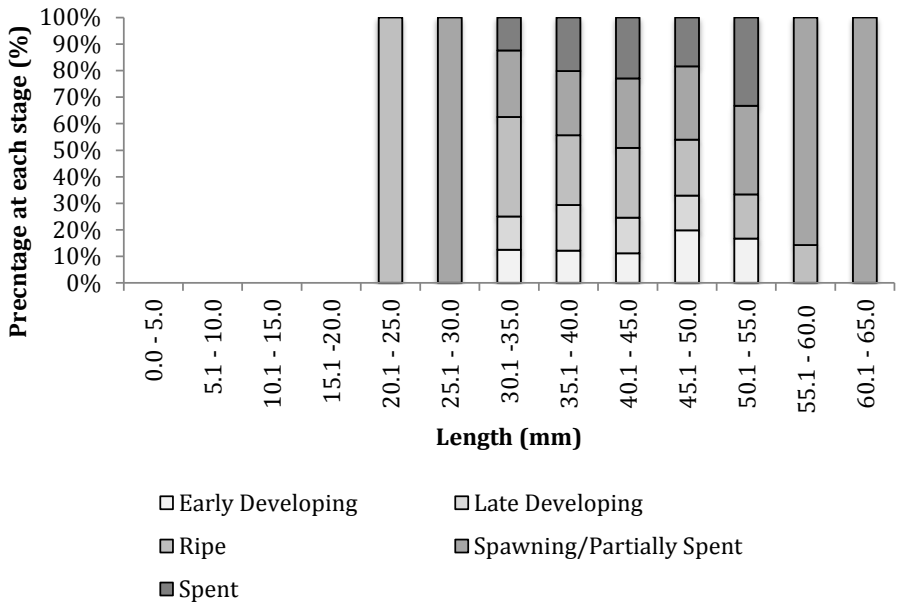
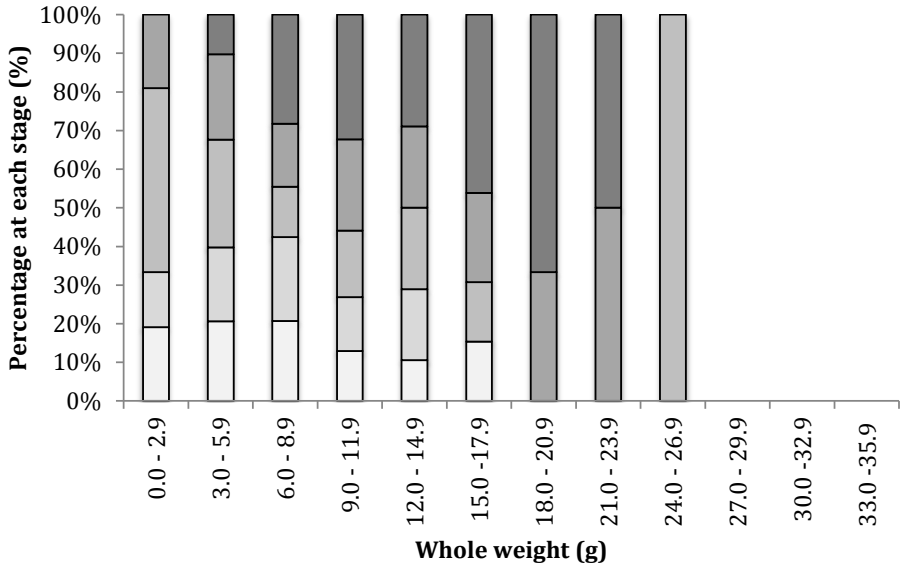


Figure 8: Percentage at each stage of gonadal maturity per length class (mm) at (a.) Bannow Bay and (b.) Flaxfort Strand.

a.



b.

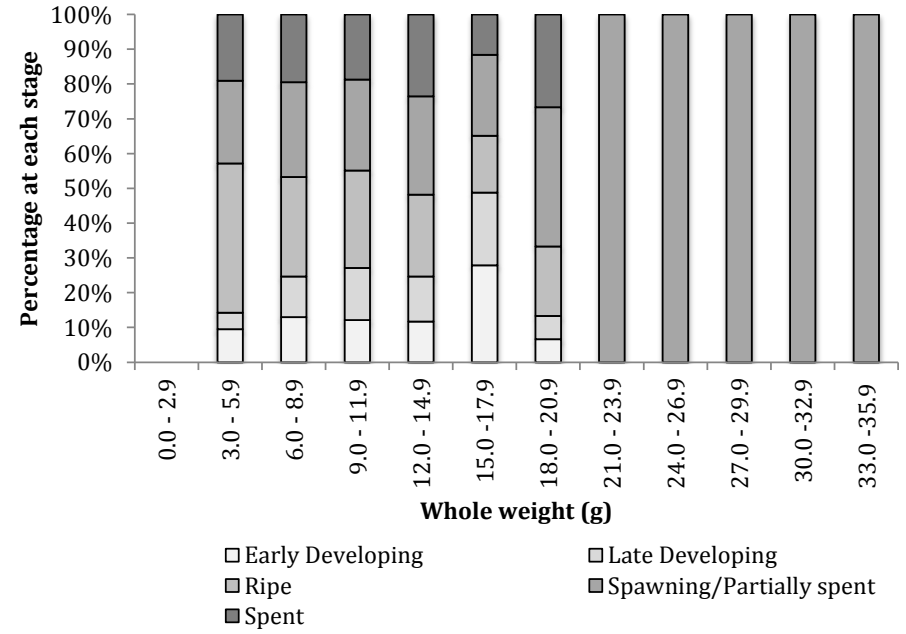


Figure 9: Percentage at each stage of gonadal maturity per weight class (g) at a. Bannow Bay and b. Flaxfort Strand.

Gonads were apparent in mussels of length 14.9 mm and upwards in Bannow Bay and 22.3 mm and upwards from Flaxfort Strand and in those with a whole weight for 0.77 g from Bannow Bay but 3.13 g from Flaxfort Strand. Differences were apparent between sites in terms of gonadal maturity, length and whole weight (Figures 8 and 9; Table 4).

Table 4: Summary of gonadal development in relation to length (mm) and whole weights (g) of *Mytilus edulis* from Bannow Bay and Flaxfort Strand.

Bannow Bay		**Flaxfort Strand**	
Length of mussels with active gonads			
%	Length (mm)	%	Length (mm)
37.5	35.1 - 40.0	36.4	40.1 - 45.0
26.1	40.1 - 45.0	28.8	35.1 - 40.0
18.9	30.1 - 35.0	20.9	45.1 - 50.0
Length (mm) of spawning mussels			
%	Length (mm)	%	Length (mm)
33.8	35.1 - 40.0	34.8	40.1 - 45.0
23.5	40.1 - 45.0	25.0	35.1 - 40.0
22.1	30.1 - 35.0	21.9	45.1 - 50.0
Whole weight of mussels with active gonads			
%	Whole weight (g)	%	Whole weight (g)
28.5	9.00 - 11.99	30.0	9.00 - 11.99
27.6	6.00 - 8.99	24.1	12.00 - 14.99

Two spawning periods were identified in mussels collected from Flaxfort Strand (April – July 2010 and September – December 2010), however only one was noted in Bannow Bay (April – July 2010). Gametogenesis occurred from late autumn and over winter at both sites, but stages of gonadal maturity were not entirely in synchrony between the sexes at each site when the study began in March 2010.

4.3.5 *Variation in gonadal development between years*

Ripe gonads were observed in male and female mussels from Bannow Bay when the study began in March 2010 which corresponded to an air temperature of 6.0 °C, however in 2011 ripe gonads were apparent from January (mean monthly water temperature = 5.66 °C, mean monthly air temperature 4.7 °C) for males and from February (water temperature 8.15 °C, air temperature 7.2 °C) for females. Spawning in 2010 at Bannow Bay occurred in April (air temperature 9.0 °C) in males and June (water temperature 17.98 °C, air temperature 14.7 °C) for females, but was three months earlier in 2011 for both, following the unusually cold winter occurring in January for males (water temperature 5.66 °C, air temperature 4.7 °C) and March for females in 2011 (water temperature 9.13 °C, air temperature 7.0 °C).

Variation in ripening and spawning times between years was also apparent in Flaxfort Strand. Ripe gonads were identified among both sexes in April 2010 (water temperature 9.55 °C, air temperature 8.2 °C) and again in October for males (water temperature 13.2 °C, air temperature 10.4 °C). Spawning was initiated in April 2010 for males (water temperature 9.55 °C, air temperature 8.2 °C) and a second spawning period was apparent in September 2010 (water temperature 15.68 °C, air temperature 13.3 °C). Females spawned in June 2010 (water temperature 17.14 °C, air temperature 14.5 °C) and November 2010 (water temperature 9.27 °C, air temperature 5.6 °C). However in 2011 spawning for both sexes occurred from March (water temperature 9.23 °C, air temperature 6.5 °C).

4.4 DISCUSSION

During the course of this research over 400 mussels from each of the two sites were screened to ascertain differences in growth, sex ratio, and gametogenesis. Differences were apparent between sites with two spawning periods in Flaxfort Strand, compared to one in Bannow Bay. The lengths, weights, and condition indices of the animals between sites varied, as did the minimum size at which mature individuals were observed. The possible potential effect of climate was identified also during 2011, as there was earlier spawning and of greater duration at both sites after a severe winter, and in addition males and females became synchronised after

the cold spell. The spawning period was extended too following the cold winter. Slightly different latitude and position on the shore, combined with food supply, were also factors that the author believes led to two spawning periods in Flaxfort Strand, compared to one in Bannow Bay.

Mean air or water temperature did not vary significantly between sites; water temperature at both was cyclical in nature, with highs in June of both years and lows in December. Mean monthly temperatures in 2011 were lower than the corresponding months in 2010. Despite a general warming trend in Europe, winter 2010, was characterised in Europe and the east coast of the United States by extreme cold, in addition to a period of extended snow (Vicente-Serrano *et al*., 2011). Concurrently the North Atlantic Oscillation (NAO) had its most negative value, for the duration of which there are data, plus the negative values of the Arctic Oscillation (AO) index were unprecedented (Vicente-Serrano *et al*., 2011). The drivers of this phenomenon are as yet unknown, however the strong phase of ENSO (El Niño Southern Oscillation) may have played a part, as it was the fifth strongest phase since 1950, and the strongest since 1997-1998 (D'Arrigo *et al*., 2011; Vicente-Serrano *et al*., 2011).

Mussels were significantly greater in all size parameters measured from Flaxfort Strand. This may be because mussels collected from Flaxfort Strand were collected at mid shore, whereas those from Bannow Bay were found on the upper shore, but these were the areas where they occurred abundantly at the respective sites. Growth rate is correlated with position on the shore and animals on the upper shore may experience prolonged periods of hypoxia which decreases the metabolic rate and thus growth (Sukhotin and Pörtner, 2001). Differential abilities to feed based on shore location, and site differences may have influenced growth rates. Growth rates in marine invertebrates have been positively correlated with temperature in their distributional range but Bannow Bay was slightly warmer than Flaxfort Strand (Thompson, 1984).

Despite being smaller in size, a greater number of mussels from Bannow Bay had gonads -if there are slower growth rates in mussels in Bannow Bay it may mean that they reach a greater age at a smaller size hence the greater presence of gonads.

Development of gonads may be related more to age than size (Kautsky, 1982) and *Mytilus edulis* can become sexually mature in their first year, and the size at which maturity is achieved can vary depending on local conditions for growth (Seed and Brown, 1977). For gonad ripening food may be as important as temperature (de Vooys, 1999). Gonads were apparent, in mussels of differing length and size at both sites but in particular in Bannow Bay at very small weights – less than 1 g. According to Kautsky (1982) gonads have been recorded in mussels of length 2.1 mm from the Baltic, and 2 mm from slow growing mussels in the Atlantic. The majority of spawning mussels from both sites were of intermediate size though slightly larger at Flaxfort. The biomass of gametes produced increases to a maximum in intermediately sized animals (Kautsky, 1982). There appears to be reliance in Bannow Bay on one or two length classes to reproduce but a greater range of animals spawned in Flaxfort Strand. Alternatively mussels from Bannow Bay could be a young population which is just reaching sexual maturity.

There was a near absence of very small mussels at Flaxfort Strand. This may be indicative of greater environmental problems at Flaxfort Strand, since mussels may be under stress and stressed adults produce less viable larvae (for succesful fertilisation) (Bayne *et al*., 1978). In a study of *Cerastoderma edule* gametogenesis which ran simultaneously at both sites, there was a paucity of younger smaller cockles too at Flaxfort Strand (Morgan *et al*., 2012). Nearby Baltimore Bay has high levels of tributyltin (TBT) and polycyclic aromatic hydrocarbons (PAHs) and there are areas which receive sewage outputs from large towns close by e.g. Kinsale and Clonakilty (Widdows *et al*., 2002). However there are outflows too in proximity to Bannow Bay, at New Ross, Rosslare and Wexford (Widdows *et al*., 2002). There can be substantial growths of *Enteromorpha* spp. at Flaxfort Strand and its surrounding areas which are not present in Bannow Bay and its water quality is known to be less good than that of Bannow Bay also (www 7; www5; Morgan *et al*., 2012). The combination of these may impact negatively on the viability of mussel larvae and therefore less recruitment to the area may occur. Additionally Flaxfort Strand is quite open, especially in comparison to Bannow Bay (Cotter *et al*., 2010)

and larvae may not be retained in the bay. Similarly the utilisation of Flaxfort Strand as a nursery ground may mean that smaller mussels are predated upon by fish.

Condition index is used as a proxy for commercial quality, or health of an animal (Okumuş & Sterling, 1998; Orban *et al*., 2002). There are many sources of variation in condition index such as season, latitude, water temperature, food and gametogenesis and wet weight, which was used, is more variable than dry weight (Pérez-Camacho *et al*., 1995; Okumuş and Sterling, 1998; Orban *et al*., 2002). There was no significant difference between condition indices (CI) between sites; but it was less at Flaxfort Strand. Perhaps the reduced condition index at Flaxfort Strand in comparison to Bannow Bay also reflects the lower water quality and greater anthropogenic inputs experienced at this site.

Males were more common at both sites, but not significantly so, but there was a greater number of mussels of indeterminate sex (post spawning) at Bannow Bay than at Flaxfort Strand. In Kautsky's (1982) study of mussels from the Baltic there was a greater number of females (49%) than males (39%), mussels of indeterminate sex were observed too (12%) plus one hermaphrodite. A greater number of female mussels were sexed in a study from Whitsand Bay, England (Secor et al., 2001). A 1:1 ratio was observed also in a study of *Mytilus edulis* from the Long Island Sound, US (Hilbish and Zimmerman, 1988) and from the Gulf of Finland (Sunila, 1981). However two hermaphrodites were recorded in the Gulf of Finland (Sunila, 1981) but none were identified in the present study.

Gametogenesis occurred over late autumn to early winter in both sites; it may have been initiated by the decrease in temperature, as has been seen in a population of Baltic mussels (Kautsky, 1982). A previous study of mussel gametogenesis in Strangford Lough, Northern Ireland documented redevelopment over winter and early spring (Seed and Brown, 1977). Gametogenesis over winter is common in *Mytilus edulis* however it began earlier in the present study than has been recorded in other studies (Table 5). At both sites in 2010 males and females were not in complete synchrony during spawning, however in 2011 after a cold period, they were. Hilbish and Zimmerman (1988) reported no difference in the rate of maturation between male and female mussels from the Long Island Sound, US,

whereas Kautsky (1982) noted that males mature faster but spawn later than females. Reproductive synchrony in terms of spawning is beneficial for a species; it means that the greatest amount of egg fertilisation will occur and that the greatest number of larvae will survive their time in the plankton (de Vooys, 1999). A swift change in temperature over a short time period may coordinate spawning (de Vooys, 1999) and Dabouineau and Ponsero (2009) believe cold winters may ensure that the sexes spawn in unison. Although gametogenesis occurred earlier than previously reported, ripening and spawning were in line with other studies.

Table 5: Gametogenesis and spawning in *Mytilus edulis* throughout its range.

Latitude	Location	Method of Assessment	Gametogenesis	Ripe	Spawning	Resting	Reference
60°06'48. 45" N	Askö Laboratory, Swedish Baltic Coast	Histology	October/November begins	Spring		August	Kautsky, 1982
59°44'24. 94" N	Tvärminne Zoological Station, Gulf of Finland	Gonad Index	November - January	February	Peaks July	August - October	Sunila, 1981
55°09'32. 45" N	Lough Foyle, Ireland	Gonad Index	October-April/March	April	April-August		McKenzie, 1986
54°28'58. 80" N	Strangford Lough, Ireland	Mean Gonad Index		Spring			Seed and Brown, 1977
54°15'29. 99" N	Dundrum Bay, Ireland	Histology	November -April	Spring	May - November	Late summer and Autumn	Snodden and Roberts, 1997
54°14'40. 39" N	Filey-Brigg, Yorkshire, England	Histology	Winter	Early spring/June	March-May	August-September	Seed, 1969
53°16'09. 41" N	Anglesey, Wales	Stereological Analysis/ Volume fraction	Autumn/Winter		Late Spring		Lowe *et al*., 1982
53°15'29. 67" N	Galway Bay, Ireland	Gonad Squash Preparati Gonad Index	Autumn-Winter	Spring	Spring and late Summer	August	King *et al*., 1989
51°45'57. 27" N	Beggar's Island, England	Stereological Analysis/ Volume Fraction		February	April	Spring and Autumn	Lowe *et a*l., 1982
40°04'29. 9" N	Long Island Sound, US	Gamete Volume Fractio	January - April	April - June	June	September/October	Hilbish and Zimmerman, 1988
40°04.29.44" N	Long Island, US	Gamete Volume Fractio	Winter	April and May		September/October	Newell *et al*., 1982

There was one spawning period in Bannow Bay but two in Flaxfort Strand, the second spawning period involved fewer animals than the first. In previous studies of *Mytilus edulis* reproduction in Galway Bay, Ireland, spring and summer peaks in spawning have been noted (King *et al*., 1989). Variations in spawning times have been reported throughout European populations of *M*. *edulis* (Lowe *et al*., 1982; Cárceres-Martínez and Figueras, 1998; de Vooys, 1999). Two spawning episodes have generally been reported in Great Britain and Ireland previously, in March/April and then in July/August (de Vooys, 1999). In contrast one spawning episode has been reported from the north of Britain, while two in the south (Lowe *et al*., 1982). If a second spawning period occurs in autumn, the following spring spawning may happen later if there are not necessary food resources available (Lowe *et al*., 1982), however this was not the case in Flaxfort Strand in 2011 as spawning began earlier for both sexes than in 2010. This may indicate that there are sufficient resources to fuel repeated spawning at Flaxfort Strand and while mussels were from lower on the shore, they may be less stressed by air exposure, and an absence of younger recruits may mean less competition for resources such as food and space. Late spawning has been described as a reproductive "risk" as mortalities may occur in animals that do not have satisfactory energy reserves, so it will only be successful if there are adequate reserves for basal metabolism and gametogenesis over winter (McKenzie, 1986).

In the present study there was an interval of 4 weeks between recorded spawning for males and 12 weeks for females at Flaxfort Strand. Although two spawning periods were observed, ripe females were not seen in our samples during the second period therefore gametogenesis for the second spawning period may have been rapid. A delay of 4 – 8 weeks between spawning periods was noted in a study in Carlingford Lough (King *et al*., 1989). Spermatogenesis in the mussel, *Mytilus californianus* can be completed within ten days (McKenzie, 1986). The second spawning period in Flaxfort Strand occurred from September (males) and November (females) until December. This is later than has previously been reported for this species. In a study in Dundrum Bay spawning ceased in November (Snodden and Roberts, 1997), and generally *Mytilus edulis* have been seen as resting or undergoing gametogenesis at this time of year. Food ability

and time taken to build up sufficient gonads to spawn could have delayed spawning.

In this study, the timing of ripeness and initiation of spawning among both sexes and at each of the sites came forward after a severe winter, by three months for males and two months for females at both sites. The spawning duration doubled in Bannow Bay, from three months for males in 2010 to six months, and in the case of females from two to four months. While in Flaxfort Strand, males spawned for an extra month and females for an additional two months. Spawning was still continuing when the study ended. Greater increases in spawning duration in Bannow Bay compared to Flaxfort Strand may be related to the fact that mussels from the former site had only one, albeit long spawning while there were two in Flaxfort Strand. Additionally greater condition index in mussels from Bannow Bay may indicate that they can sustain a longer spawning period. Over the winter months mussels from Flaxfort Strand may not have acquired sufficient reserves to allow for an early protracted spawning in 2011. Advancement and extension of the spawning period among mussels mirrors the findings of two previous studies at Bannow Bay and Flaxfort Strand, on the soft shell clam (*Mya arenaria*) and the common cockle (*Cerastoderma edule*) (Cross *et al*., 2012; Morgan *et al*., 2013). In the case of *C*. *edule*, advancement and extension of the spawning period was linked to alterations in phytoplankton availability, and attempts to buffer the potential effects of climate change by trickle spawning to ensure at least some larvae coincide with the spring plankton blooms (Morgan *et al*., 2013). Although cockles and mussels have contrasting reproductive strategies, cockles being income breeders while mussels are conservative, the same principle may hold in this case. Mussels due to their plasticity may alter their reproductive strategy, and as previously stated a range of reproductive approaches may exist, subject to ambient conditions (Newell *et al*., 1982; Bayne *et al*., 1983). For example in the Baltic, gonad ripening was seen at 1-3 °C and was related to spring blooms (Kautsky, 1982) but other studies state that spawning only occurs above 10 °C (de Vooys, 1999). In the present study spawning was on-going at 9.0 °C. Yearly variance in gametogenic cycle may be related to contemporary nutrient availability and temperature.

Although gametogenesis began earlier in the two years, spawning occurred at roughly the same time. This may indicate that whichever process is altering commencement of gametogenesis thus far has not had the same effect on spawning. Gametogenesis may be induced by reduction in temperature or by reaching a "critical" amount of nutrient reserves (Hilbish and Zimmerman, 1988). Spawning is governed by various endogenous and exogenous factors which are as yet not fully understood (food availability, temperature, photoperiod). Duration and instigation of spawning were altered by a period of extreme cold, starting earlier and lasting longer. This may lead to a greater level of *Mytilus edulis* larvae and perhaps greater recruitment. However this severe winter was anomalous, and did not adhere to the current trend of warming winters. If colder winters are getting rarer, the spawning period in mussels may be somewhat curtailed, perhaps leading to a reduction in mussel biomass. This could be exacerbated by competition with fisheries for seed, and fishing pressure could deter recovery if mussels do decline. The fact that there were two spawning periods in Flaxfort Strand, but only one in Bannow Bay, coupled with the smaller size of mussels from Bannow Bay, may indicate greater opportunity to accrue nutrients in Flaxfort Strand due to competition, position on the shore, and allochthonous inputs.

In conclusion, a number of differences in mussel population dynamics and gametogenesis were apparent between sites and years. These variations may be due to site dissimilarities in food availability, water temperature, position on the shore, exposure, turbidity and currents. Interannual variation was apparent in spawning times, and this may be linked to a severe winter and the warm summer that preceeded it. Gametogenesis occurred earlier than has been reported previously. However if this period of warmer conditions continue in the Irish Sea, albeit with occasional colder winters, there may be declines in recruitment. This may mean that consistently strong reproduction may not be possible, and mussel biomass may decline. If this does occur, it could lead to a reduction in mussel production, and disputes between commercial interests versus conserving what larvae and spat there are to create the natural beds that many other organisms rely upon.

SECTION 2: Comparison of *Cerastoderma edule* and *Mytilus edulis* gametogenesis, gonadal maturity and spawning times.

Table 6: Gonadal production, ripeness and spawning of *Cerastoderma edule* and *Mytilus edulis*.

	Male		**Female**	
	2010	2011	2010	2011
Cerastoderma edule				
Bannow Bay				
Gametogenesis	March - April	September - May	March - April	October - February
Ripe	April - September	March - May	April - November	March - June
Spawning/Partially Spent	June - November	February - June	March - April	March - June
Flaxfort Strand				
Gametogenesis	April	October - April	April - May	October - May
Ripe	April - August	March - June	April - September	March - June
Spawning/Partially Spent	May - August	April - June	May - September	April - June
Mytilus edulis				
Bannow Bay				
Gametogenesis	March - April	September - February	March - May	August - March
Ripe	March - June	January - April	March - June	February - April
Spawning/Partially Spent	April - June	January - June	June - July	March - June
Flaxfort Strand				
Gametogenesis	n/a	September - January	April - May	August - February
Ripe	April - June October	October - April	April - June	February - May
Spawning/Partially Spent	April - July September - December	March - June	June - July November - December	March - June

Gametogenesis in cockles began over winter and continued until late spring/early summer (Table 6). Gametogenesis began in mussels at approximately the same time, but was completed earlier in spring. Ripe gonads were observed in cockles from spring to autumn and spawning continued until winter. However in mussels ripe gonads were apparent in spring/early summer and again in winter in Flaxfort Strand only. Spawning occurred from spring to summer, and again in late winter in mussels.

SECTION 3: Biology and reproduction of *Macoma balthica*, *Angulus tenuis*, and *Scrobicularia plana*.

4.5 ABSTRACT

Increasing attention is being turned to synecology – the study of whole animal communities. Within soft sediment communities, bivalves with commercial importance e.g. *Cerastoderma edule* have been given more attention than little known but ecologically important bivalves such as *Macoma balthica* (Baltic tellin), *Angulus tenuis* (thin tellin) and *Scrobicularia plana* (peppery furrow shell). The present study is the first investigation of the population structure and reproductive biology of these aforementioned bivalves in Ireland. Samples were collected from Flaxfort Strand, Co. Cork and Bannow Bay, Co. Wexford over a 16 month period, from March 2010 to June 2011. Both sites are Special Areas of Conservation and Special Protected Areas under the EU Habitats and Birds Directives. Constant temperature loggers were deployed at each site too, and the temperature was recorded every 16 minutes. A series weights and measurements were taken, before the bivalves were processed for histology.

4.6 INTRODUCTION

Alterations in the thermal regime, salinity, pH and sea level rise, as a result of climate change, will have concurrent effects on all organisms in the marine environment, by affecting individuals which can feed into populations and impacts at community level (Roessig *et al*., 2004; Fujii, 2012). Physiology, behaviour, growth, fecundity and recruitment of species will all be influenced by climate change (Fujii, 2012). The impact may be expressed primarily in near coastal and estuarine areas, as they are shallow and have rapid temperature changes which will affect their communities (Madeira *et al*., 2012). Indeed a succession of low bivalve recruitment (*Cerastoderma edule, Macoma balthica, Mya arenaria*) in the Wadden Sea had been attributed to a series of mild winters (Philippart *et al*., 2003). It has been hypothesised that as ectotherm metabolism decreases at low temperatures, they have a greater preservational biomass and can produce a greater number of eggs leading to greater recruitment after a particularly cold winter (Honkoop and van der Meer, 1998; Philippart *et al*.,

2003). However studies using *M. balthica* have shown that lower egg production after a mild winter only accounts for 7% of the variation in recruitment between years (Flach, 2003; Beukkema *et al*., 2001; Beukkema and Dekker, 2005). Studies in Sweden and the Dutch Wadden Sea suggest that a predator prey mismatch, as a result of increasing winter temperatures, may play an integral part in recruitment success or failure (Beukkema *et al*., 2001; Beukkema and Dekker, 2005). Post larvae are predated upon by green crabs *Carcinus maenas* and shrimps *Crangon crangon* and their arrival on the tidal flats can be delayed following a cold winter and they are present in lower numbers too (Flach, 2003). This can lead to a predator prey mismatch between these epibenthic predators and bivalve post larvae (Strasser and Gunther, 2001; Strasser, 2002; Flach, 2003; Strasser *et al*., 2003; Beukkema and Dekker, 2005). Additionally it has been hypothesised that post larvae, in the interim, reach a certain "refuge size" before the arrival of their predators again reducing predation pressure upon them (Strasser and Gunther, 2001; Flach, 2003).

Several species were focused on in this study of which little or nothing is known of their biology and reproduciton in Ireland: the Baltic tellin (*Macoma balthica*), the thin tellin (*Angulus tenuis*), and the peppery furrow shell (*Scrobicularia plana*). *Macoma balthica,* the Balthic tellin, is a common bivalve from Norway to Arcahon Bay, France in Europe and from Greenland to South Carolina in the US (Beukema and Meehan, 1985; Hummel *et al*., 1996; Kamermans *et al*., 1999; Jansen *et al*., 2007; Long *et al*., 2008). It can form dense aggregations and can be a food source for birds and crabs (Griffith and Richardson, 2006; Long *et al*., 2008). *M. balthica* alternates between suspension and deposit feeding (Griffiths and Richardson, 2006). The number of spawning events is latitude dependent: in northern locations, they spawn in spring, while further south in France spring spawning is followed by redevelopment and further spawning in September/November (Günther *et al*., 1998).

Angulus tenuis (= *Tellina tenuis*) the thin tellin is a suspension feeder found in in sandy, sheltered beaches (Dekker and Beukema, 1999). It uses a long siphon to feed, and this siphon may itself be removed by feeding fish (Trevallion, 1971).

Scrobicularia plana, the peppery furrow shell, is a widespread estuarine bivalve. It is distributed from Norway to Senegal, and into parts of the Mediterranean (Rodriguez-Rua *et al*., 2003). *S*. *plana* is a deposit and suspension feeder, preferentially inhabitting muddy sediments (Rodriguez-Rua *et al*., 2003; Chesman and Langton, 2006; Santos *et al*., 2011).

The present study aimed to investigate the population structure, biology and gametogenesis of several little studied marine bivalves and to compare them across two sites. The current study is the first of its kind in Ireland, and no previous studies of *Macoma balthica* or *Angulus tenuis* reproduction in Ireland exist.

4.7 RESULTS

Macoma balthica, *Angulus tenuis* and *Scrobicularia plana* were found in the greatest numbers at both sites. There were higher densities apparent in Flaxfort Strand than Bannow Bay (Table 7).

4.7.1 *Macoma balthica*

Macoma balthica from Flaxfort Strand were greater in all parameters measured than those from Bannow Bay and this was significantly so for height, width, whole and shell weights (Table 8). There was a greater number of male *Macoma balthica* at Bannow Bay, but at Flaxfort Strand the situation was reversed (Figure 10).

Three stages of gonadal development were seen in male and female *Macoma balthica* over the course of the study; developing, ripe/spawning and spent. Males from Bannow Bay were developing (100 %) in April 2010 and were again developing in February 2011 (50 %) and March 2011 (14.3 %). Ripe/spawning males were apparent from November 2010 (100 %) to January 2011 (100 %) and then in March 2011 (87.5 %). Spent males were observed in June 2010 (100 %) and March 2011 (14.3 %). Females from the same site were ripe/spawning in July 2010 (14.3 %) and some were in spent condition in the same month (58.7 %). Redevelopment of gonads occurred from February 2011 (42.9 %) (Figure 13).

Males from Flaxfort Strand were developing from July 2010 (20.0 %) until September 2010 (100.0 %). Spawning began October 2010 (100.0 %) until December 2010 (100 %). Spent males were identified in June 2010 (100.0 %). Female *Macoma balthica* from Flaxfort Strand, were developing from July 2010 (14.3 %) until September 2010 (50 %). They were ripe/spawning from October 2010 (20.0 %) until December 2010 (33.3 %). Redevelopment of gonads was observed from December 2010 (66.7 %) (Figure 11).

4.7.2 *Angulus tenuis*

Angulus tenuis was larger in terms of length, width, height, weights at Bannow Bay than Flaxfort Strand. There was significantly more male *A. tenuis* at Bannow Bay however, but at Flaxfort Strand there was no significant difference and there was a greater number of females (Figure 10).

Thin tellins were organised using four stages of gonadal development: developing, ripe, spawning/partially spent and spent (Figure 12).

In April 2010, the majority of males from Bannow Bay were late developing (80.0 %) and a lesser number were ripe too in April 2010 (20.0 %). Spawning began in May 2010 (11.1 %) and continued until July 2010 (100 %) when the last *Angulus tenuis* were encountered. Females from Bannow Bay were developing in April 2010 (100 %), they began spawning in May 2010 (100 %) and this continued until July 2010 (100 %) (Figure 14).

Male *Angulus tenuis* from Flaxfort Strand were ripe from June 2010 (25.0 %) until July 2010 (37.5 %). Spawning began in June 2010 (75.0 %) and they continued to spawn until August 2010 (80.0 %). Spent males were recorded in August 2010 (20.0 %). Females too were in ripe condition from June 2010 (25.0 %) to July 2010 (66.7 %). Females spawned from June 2010 (68.8 %) until August 2010 (50.0 %). Spent females were recorded from August 2010 (50.0 %) (Figure 12).

4.7.3 *Scrobicularia plana*

Scrobicularia plana collected from Bannow Bay however were larger (length, width, height, weight) than the animals from Flaxfort Strand. There was a greater number of female *Scrobicularia plana* at Bannow Bay, but equal numbers of males and females at Flaxfort Strand (Figure 11).

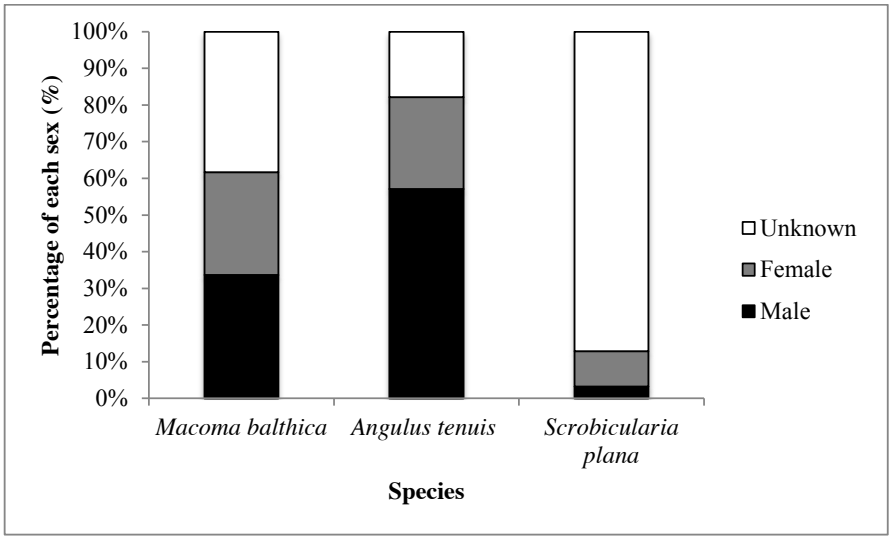
Table 7: Number of specimens of each species collected. (N/S = no sample, due to adverse weather conditions).

	Bannow Bay					**Flaxfort Strand**			
	Macoma balthica	*Angulus tenuis*	*Scrobicularia plana*	*Mya arenaria*	*Ruditapes* spp.	*Macoma balthica*	*Angulus tenuis*	*Scrobicularia plana*	*Mya arenaria*
Mar-10	0	0	20	0	0	n/s	n/s	n/s	n/s
Apr-10	9	11	2	0	0	0	2	4	0
May-10	1	21	0	0	0	16	15	0	0
Jun-10	30	12	0	0	0	30	30	2	1
Jul-10	2	5	0	0	0	30	12	3	0
Aug-10	0	0	0	0	0	18	10	3	0
Sep-10	0	0	2	0	0	5	0	5	0
Oct-10	0	0	0	0	0	30	5	2	0
Nov-10	9	3	3	0	0	5	3	2	0
Dec-10	n/s	n/s	n/s	n/s	n/s	7	8	0	0
Jan-11	5	0	0	4	0	1	1	1	0
Feb-11	12	0	2	0	1	0	2	0	0
Mar-11	20	0	0	0	0	2	0	0	0
Apr-11	15	0	0	1	0	24	4	0	0
May-11	0	0	0	0	0	16	0	0	0
Jun-11	4	4	0	0	0	12	0	0	0
Total	107	56	31	5	1	193	92	22	1

Table 8: Measurements and ranges taken for each bivalve from Bannow Bay and Flaxfort Strand combined.

	Macoma balthica		***Angulus tenuis***		***Scrobicularia plana***	
	BB	FF	BB	FF	BB	FF
Mean Height (mm)	12.8 ± 2.08	14.5 ± 0.72	11.7 ± 1.38	11.4 ± 1.24	22.5 ± 3.86	18.2 ± 1.90
Range	5.6 - 20.5	7.2 - 19.1	4.9 - 14.5	7.9 - 18.2	14.9 - 36.7	12.4 - 22.2
Mean Length (mm)	15.1 ± 1.53	16.4 ± 2.49	16.6 ± 2.1	14.9 ± 4.13	21.2 ± 10.72	21.6 ± 8.01
Range	5.3 - 20.4	8.1 - 21.9	8.4 - 21.5	2.6 - 22.4	6.4 - 36.7	4.1 - 29.3
Mean Width (mm)	6.7 ± 1.27	7.9 ± 0.64	3.9 ± 0.98	3.7 ± 0.55	7.8 ± 1.43	7.0 ± 0.77
Range	1.9 -18.0	3.1 - 10.7	0.9 - 5.0	2.4 - 6.8	6.2 - 21.8	4.3 - 8.9
Mean Whole Weight (g)	1.0 ± 0.27	1.5 ± 0.35	0.6 ± 0.09	0.5 ± 0.11	3.5 ± 1.53	1.7 ± 0.41
Range	0.1 - 2.4	0.1 - 2.9	0.1 - 1.0	0.2 - 1.8	1.1 - 9.8	0.5 - 2.8
Mean Tissue Weight (g)	0.2 ± 0.05	0.4 ± 0.33	0.2 ± 0.04	0.2 ± 0.06	1.2 ± 0.18	0.5 ± 0.1
Range	0.04 - 0.4	0.02 - 0.9	0.02 - 0.4	0.04 -0.4	0.3 - 2.9	0.1 - 0.9
Mean Shell Weight (g)	0.6 ± 0.18	0.9 ± 0.27	0.3 ± 0.05	0.2 ± 0.06	1.5 ± 0.56	0.5 ± 0.13
Range	0.04 - 1.6	0.07 - 1.9	0.02 - 0.5	0.07 - 0.7	0.4 - 4.3	0.2 - 1.0

(a.)



(b.)

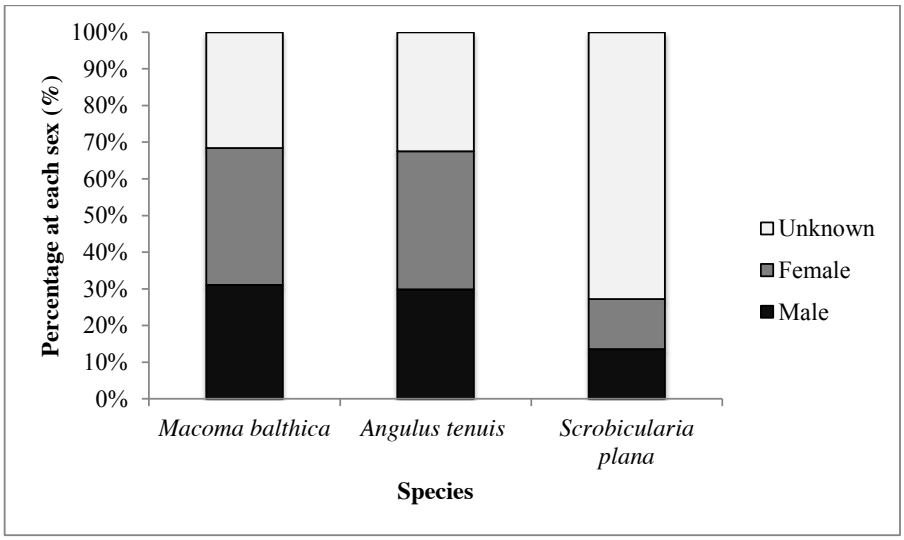


Figure 10: Sex ratio in species collected at (a.) Bannow Bay and (b.) Flaxfort Strand.

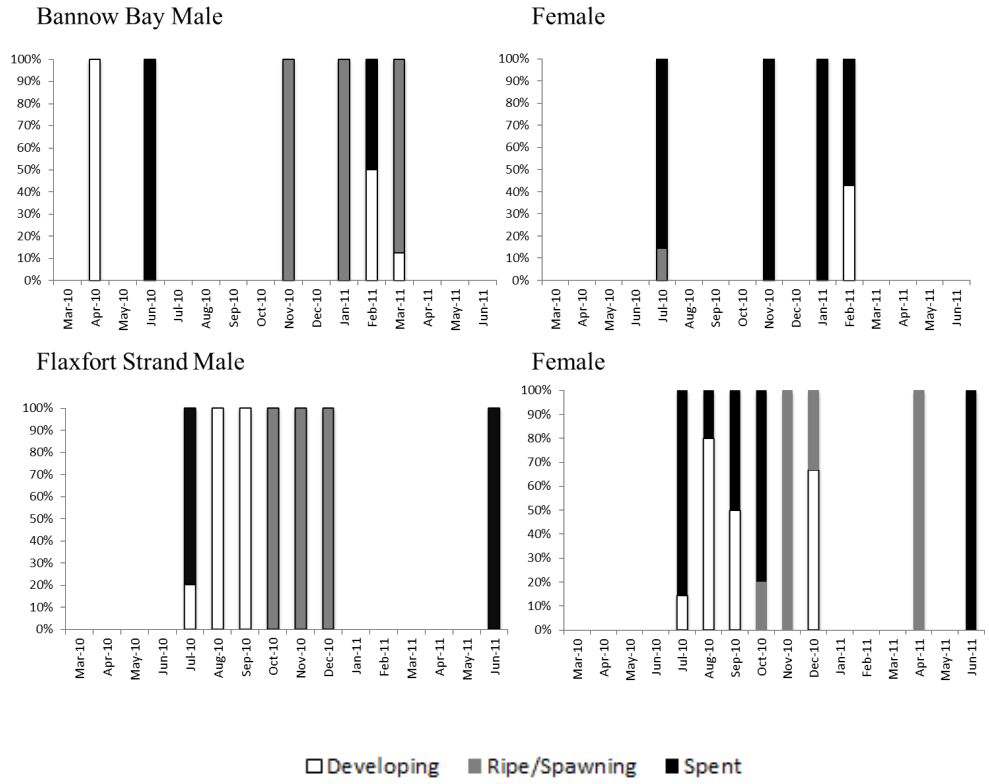


Figure 11: Percentage of animals at each stage of gonadal maturity in male and female *Macoma balthica* sampled over the study period from Bannow Bay and Flaxfort Strand.

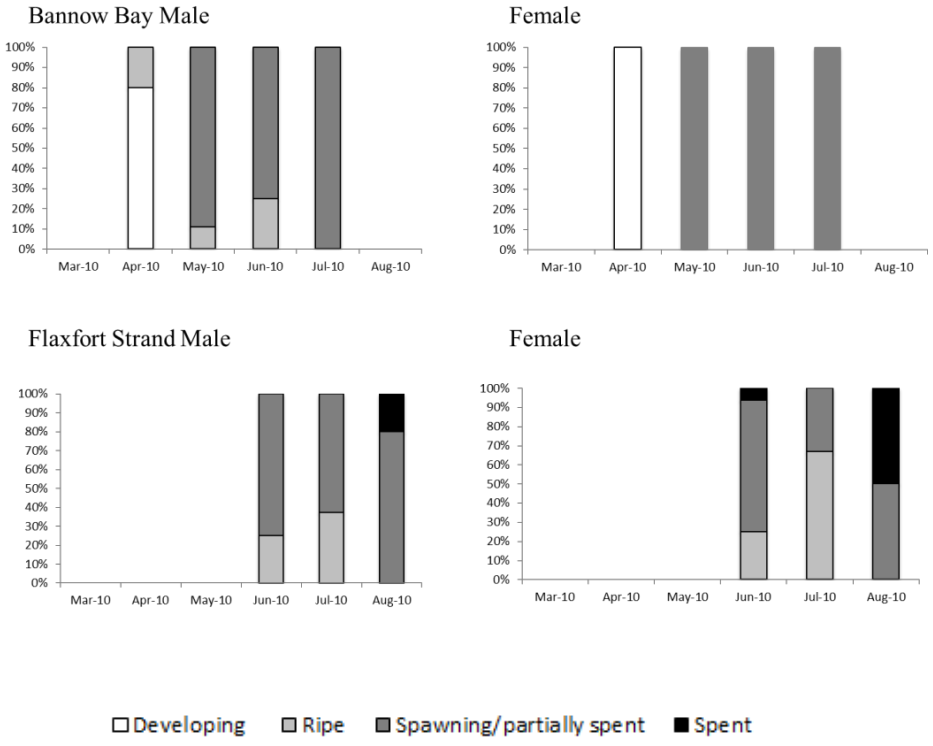


Figure 12: Percentage of animals at each stage of gonadal maturity in male and female *Angulus tenuis* sampled over the study period from Bannow Bay and Flaxfort Strand.

4.8 DISCUSSION

This present study aimed to explore the biology and the health of lesser considered bivalves in Ireland. The species under study may interact as they exploit the sediment, the same food source (plankton) and may be predated upon by crabs, fish and birds (Table 9).

Table 9: Summary of main results.

Biology
Macoma balthica was the most abundant bivalve at each site.
Greater numbers of animals at Flaxfort Strand than Bannow Bay.
There was variation between sites in size and sex ratio.
Large numbers of indeterminate *M. balthica* at both sites -could be nursery area.
Reproduction
Macoma balthica developing in Spring and spawned over winter.
Angulus tenuis spawned over summer.

Sediment particle size and substratum type can contribute to the variation within and between estuaries in macrofaunal species composition, density and biomass (Fujii, 2012). The sites under study, Bannow Bay and Flaxfort Strand exhibit quite different characteristics such as size, tidal exchange, and in substratum type. Bannow Bay is composed of mud, sand, coarse and fine sands whereas Flaxfort Strand is more homogenous sand and mud flats. The differences in substratum type may relate to the differences observed in bivalve species richness and abundance between the sites. *Macoma balthica* (Baltic tellin), *Angulus tenuis* (thin tellin), *Scrobicularia plana* (peppery furrow shell), and *Mya arenaria* (soft shell clam) were collected at both sites while *Ruditapes* sp. was found in Bannow Bay only. The depth to which the sediment was excavated too may have played a part, as *M. arenaria* bury deep in the sediment. A greater range of species was observed in Bannow Bay (n = 5) than Flaxfort Strand (n=4) which could reflect the greater heterogeneity of this area. There was variation among species in terms of density; *Macoma balthica* and *Angulus tenuis* were denser at Flaxfort Strand, whereas *Scrobicularia plana* and *Mya arenaria* were more abundant at Bannow Bay. There was variation between years in the numbers and species collected at Bannow Bay. Mortalities may have occurred over the cold winter in these bivalves leading to lower densities. The abundance of each species per site may be reflective of the needs of the animal and whether their preferred substrate is common at that area. Similarly it could be indicative of successful recruitment and low levels of predation. At Flaxfort Strand in

particular there are high densities of cockles (Fermer 2009; Morgan personal observation) which may be predated upon preferentially by waterbirds as they are near the surface especially in contrast to *M. arenaria*.

In general bivalves collected from Bannow Bay were greater in measured characteristics (length, width, height, whole weight, shell weight and tissue weight) than those from Flaxfort Strand. Variation in growth and therefore size may be influenced by many factors over a large scale e.g. latitude and at a local scale such as available nutrients (Cardoso *et al*., 2007). Bannow Bay was warmer than Flaxfort Strand which may encourage higher growth rates. There were higher numbers of bivalves collected from Flaxfort Strand, and reductions in growth rates in sediment bivalves may occur at high densities due to nutrient resource depletion (Peterson and Beal, 1989). In a study of *Macoma balthica* growth rates in the Dutch Wadden Sea, differences in food availability between sites was highlighted as a source of variation (Cardoso *et al*., 2007). Extensive colonisation by the macroalgae *Enteromorpha* spp. was seen too at Flaxfort Strand particularly in the summer months which may contribute to food limitation (de Montaudouin 1996).

Greater numbers of males were observed in Bannow Bay than in Flaxfort Strand. According to Morton (1991) male biased sex ratios may be commonplace in areas with great fluctuations in the environment, and high juvenile mortality to allow for an equal sex ratio. Females were more common at Flaxfort Strand, this strategy is thought to be adopted to heighten reproductive success by investing resources into female gametes (Morton, 1991). In each bivalve species examined there was consistently a proportion of animals with no gonads apparent, in particular in *Macoma balthica*. This may be as a consequence of the positioning of the study sites in the intertidal areas. Although found in the intertidal area, this may be more of a nursery area for this tellinid, with greater recruitment occurring in the subtidal (Cardoso *et al*., 2007). Previous studies of *M. balthica* in the Dutch Wadden Sea have identified that a low percentage of adults develop gonads in the intertidal and some do not reach sexual maturity in the intertidal (Cardoso *et al*., 2007). Additionally the wide ranges of sizes of animals collected (as no minimum was imposed) may have led to the collection of sexually immature bivalves.

The reproductive strategies of *Macoma balthica* and *Angulus tenuis* were examined. Summer spawning was observed in *A. tenuis*, however ripe/spawning gonads were observed in *M.*

balthica over winter and in spring. Due to the lack of samples or other studies in Ireland it cannot be said whether this is the normal reproductive strategy of both, or if they showed variation due to the anomalous temperatures as was found in other bivalves at these sites (e.g. *Mya arenaria* Cross *et al*., 2012; *Cerastoderma edule* Morgan *et al*., 2013). There may have been repeated spawning in *M. balthica*. Spawning in *M. balthica* occurred for a longer duration that in *A. tenuis*. Prolonged spawning in *Cerastoderma glaucum* and *C. edule* has been linked to mitigating mismatches with plankton pulses (Guillou and Tartu, 1992; Morgan *et al*., 2013). Alteration in plankton phenology due to climate variability is therefore allowed for by longer spawning duration (Dabouineau and Ponsero, 2009).

In conclusion, a number of bivalves were collected from both sites, with a greater range in Bannow Bay than Flaxfort Strand. Assessing the biology of the various bivalves showed variation in the growth rates between sites which could be due to latitude, nutrient availability and macroalgal growths. Variation in the sex ratio between sites was observed also. The reproductive biology of *Macoma balthica* and *Angulus tenuis* (albeit a small number of samples) was assessed. Earlier spawning than has been reported in other sites was observed in *M. balthica* but further large scale studies are needed to investigate their reproductive biology fully.

4.9 ACKNOWLEDGEMENTS

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CHAPTER 5: EVERY COCKLE FOR ITSELF: VIEWING THE SURFACING PHENOMENON FROM A MICROCOSM APPROACH.

5.1 ABSTRACT

Since Snieszko, (1974) proposed that disease develops following a close interaction between host - parasite and environment, the view has been held that external drivers can influence parasite development within the host. To build on this a site-specific aetiology for surfacing in cockles is proposed in the present study. Mass surfacing and mortality events have been reported in the last 20 years over geographically distinct regions in the commercially and ecologically important common cockle, *Cerastoderma edule*. Across its range the bivalve is known to provide a habitat for 16 species of digenean trematodes, in addition to copepods, gregarines and various turbellaria and cilates. Various aetiologies have been proposed for surfacing in this infaunal bivalve including the pathological condition disseminated neoplasia, haplosporidia and digenean trematodes but the effects and impacts of a number of parasites, all interacting and affecting the physiology of an individual, along with external drivers of disease development in a cockle together have not been considered fully by histological and molecular means (polymerase chain reaction). The present study attempted to explore the occurrence of these surfacing events at two quite different sites in Ireland. Prevalence and intensity of any parasites or pathological conditions were then considered in the context of local conditions like site, temperature/season, and position on or in the sediment, in addition to aspects of each cockle itself (sex, gonadal maturity, number of growth rings). Results suggest that the surfacing aetiology is site specific, with surfacing at one site (Bannow Bay) being driven by a complexity of parasite infections and season being a key component, while attaining a threshold age of three years seems to be the most significant component at the second site resulting in a threshold infection load leading to disease and death in these cockles.

5.2 INTRODUCTION

The value of the estuarine ecosystem, and the range of the services that they offer are exemplified by the common infaunal bivalve, *Cerastoderma edule*. *Cerastoderma edule*, the common cockle is widely distributed from northern Europe to Mauritania in West Africa. It can be locally very abundant in sandy bays and estuaries with 60 % of the overall biomass constituting cockles, and despite its wide range it is sensitive to extremes in temperature (André *et al*.,

1999; Reise, 2003; Dabouineau and Ponsero, 2009; Cesar, 2009). In accordance with their large biomass, cockles play a vital role in the ecosystem acting as a linkage between primary producers i.e. plankton and the many other species that rely upon them as a food source (Morgan *et al*., 2013). *Cerastoderma edule* are predated upon by the crab *Carcinus maenas*, shrimp *Crangon crangon*, fish *Pleuronectes platessa* and birds, such as the oystercatcher *Haematopus ostralegus*. The aforementioned crab, shrimp and fish species are subject to commercial fisheries, as are cockles themselves, so they play a dual role performing a supporting role within the ecosystem and being a commercial interest (Cesar, 2009; Morgan *et al*., 2013). Many bird species that rely upon cockles as a food source are listed on the EU Birds Directive, for example the oystercatcher *Haematopus ostralegus*.

The cockle itself could also be considered as a microcosm of the estuarine ecosystem as a whole; providing habitat to a range of parasites including 16 species of digenean trematodes, copepods, turbellarians, gregarines, and a suite of bacteria and viruses throughout its range (de Montaudouin *et al*., 2009). Within the cockle these parasites may form an infracommunity and exploit different tissues (de Montuadouin *et al*., 2009). Within the cockle, digenean trematodes can be confined to specific tissues and areas. Many animals can harbor multiple infections of trematode metacercariae without experiencing mortalities but a loss of condition or growth reduction may be apparent (Wegeberg and Jensen 1999). However there have been consistent reports of mass surfacing and mortalities of cockles in several areas across Europe over a prolonged period of time. Surfacing itself has been described as a "prelude to death" and several causes have been proposed, dependent on location (Blanchet *et al*., 2003). These include haplosporidians (Burry Inlet, Wales) (Anonymous – DEFRA, 2011), trematode infestation (Northern Wadden Sea) (Thieltges, 2006), disseminated neoplasia (Galicia, Spain) (Carballal *et al*., 2001) and trematodes and neoplasia acting in unison (Cork, Ireland) (Morgan *et al*., 2012). Mortalities due to parasites may occur if the balance between host and parasites is altered in some way, and the alteration is unfavourable to the host, overwhelming it (Combes, 1997).

In an ecosystem, not only may parasites, in particular trematodes, exert considerable influence, they may too comprise a large portion of the biomass

(Mouritsen and Poulin, 2002; Kuris *et al*., 2008). Indeed the depth of their influence has been compared to that of a top predator (Poulin, 1999). Parasites may harm one species more than another, and within the host they share resources and habitat (Poulin, 1999; Mourtisen and Poulin 2002). Their presence in the host may result in endocrine disruption, as in the case of trematode sporocysts or host physiology (e.g. trematode metacercariae interfering with metallothionen synthesis in cockles) (Baudrimont *et al*., 2006). They may also influence interspecific competition between hosts and predator prey interactions (Mouritsen and Poulin, 2002). However the aim of a good parasite is not to exert undue harm, but to continue to live within its host environment by being moderately pathogenic, that is until the host's death is necessary for transmission (Combes, 1997). The deleterious effect of parasites may be neglible under normal or optimal conditions, but enhanced mortality might occur during unfavourable conditions (Mouritsen and Poulin, 2002).

Disease is not solely due to the host however, and external factors are of importance too (Snieszko, 1974). The influence of external factors can be seen in the case of the Pacific oysters, *Crassostrea gigas* and ostreid herpesvirus. Mortalities in *C*. *gigas*, have been reported in France, Japan, USA and Australia for the past 20 years in the summer months (Sauvage *et al*., 2009). A number of external and internal causative factors may act together to induce summer mortalities (Sauvage *et al*., 2009). Factors which have been implicated in the mortality events include; food limitation, oxygen depletion, salinity and elevated temperature (Sauvage *et al*., 2009). These environmental issues in combination with maturation stress and pathogens may result in mortalities (Sauvage *et al*., 2009).

A pathological condition - disseminated neoplasia is known too from cockles, it was first identified in Cork harbour in 1982 and later that year in Brittany, France (Twomey and Mulcahy, 1988; Twomey 1992; da Silva *et al*., 2005). Disseminated neoplasia is of unknown aetiology but a viral causal agent has been hypothesized (Romalde *et al*., 2007). It is characterized by the proliferation of pleomorphic, mitotically active haemocytes (Ciocan and Sunila, 2005; Romalde *et al*., 2007) that are incapable of phagocytosis.

The aims of this study were to document the parasite fauna of cockles at two sites separated by 215 km in Ireland. As both sites are designated under the EU

Birds and Habitats Directives, the taxon richness was of interest as parasites can be indicative of biodiversity (de Montaudouin *et al*., 2009). One objective of this part of the study was to identify potential emerging diseases, as climate variability is a significant environmental component in this region. Secondly, the study aimed to consider the phenomenon of cockle surfacing at two quite different sites and determine if the drivers of this phenomenon i.e. the underlying causes were common to both or were site specific. Thirdly, potential interactions between the host-parasite relationship and a range of external influences on this interaction was examined.

5.3 RESULTS

Of the 1696 cockles screened by histology, 91.9 % were in some way parasitised, affected by pathological conditions or morphological changes with only 138 cockles "healthy" with none of the above observed.

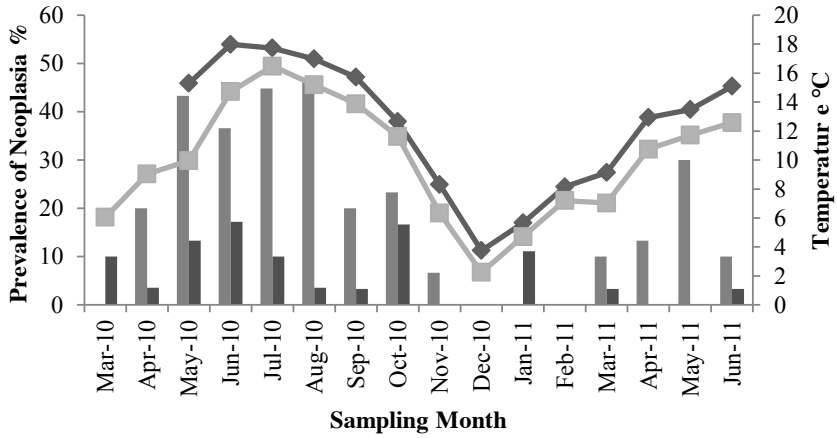
A total of fourteen parasite species were identified in cockles from Bannow Bay and Flaxfort Strand over the course of the study (Table 2, Figure 2), as was one pathological condition disseminated neoplasia plus two morphological changes (Table 4, Figure 3). Parasite species were not common to both sites, with gill cysts and cestodes present in Bannow Bay only and unidentified bacteria 2 in Flaxfort Strand only. Similarly morphological changes were not in evidence at both sites, with haemocyte infiltration of the tissues confined to Bannow Bay only. No samples were collected for December 2010, samples were collected for all other dates, if there is no data presented for a certain month it indicates that the parasite or pathology was not observed that month.

5.3.1 *Disseminated Neoplasia*

Disseminated neoplasia was diagnosed in cockles at both sites. The mean prevalence ± standard deviation of neoplasia in surfaced cockles from Bannow Bay was 21.8 ± 16.25% and in buried cockles it was 6.4 ± 6.12%. Neoplasia was significantly more prevalent in surfaced rather than buried cockles (Mann-Whitney Test P = 0.007) (Figure 2a). There was also a higher mean prevalence of neoplasia in surfaced cockles (27.1 ± 20.18%) than buried cockles (4.2 ± 5.69%) at Flaxfort Strand and this proved significant (Mann-Whitney Test P = 0.003) (Figure 2b).

In cockles from Bannow Bay, Stage 2 (n = 30) and stage 3 (n = 40) infections were the most common among surfaced cockles and were seen in significantly higher numbers than in buried cockles (Mann-Whitney Test P = 0.001; 0.029) (Figure 3). There were a greater number of stage 4 infections identified among surfaced cockles in Flaxfort Strand that Bannow Bay (FF 10: BB 3) (Figure 4). There were a significantly higher number of stage 2 and 3 infections in surfaced rather than buried cockles at Flaxfort Strand (Mann-Whitney Test P= 0.001). 44.4% of buried cockles with neoplasia at Bannow Bay had two growth rings, whereas neoplasia was most commonly encountered in surfaced cockles with three growth rings at the same site (22.7%). The highest prevalence of neoplasia was in surfaced and buried cockles with three growth rings at Flaxfort Strand (surfaced 48.6%: buried 52.9%). Stage 3 and 4 infections were most common at both sites in the summer months (Figure 3 and 4).

a.



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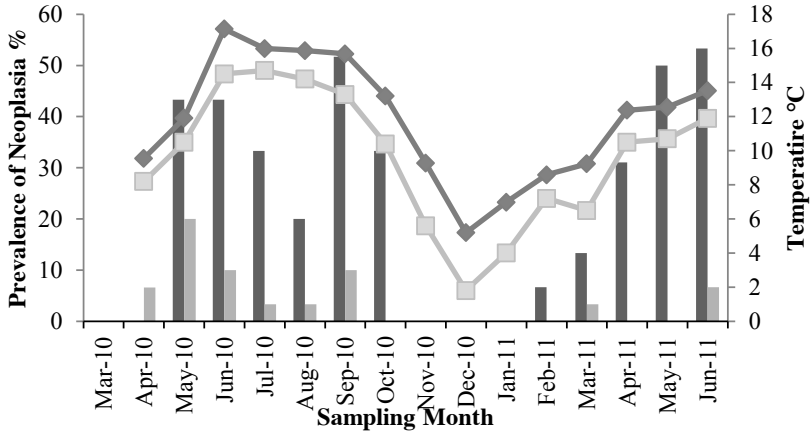


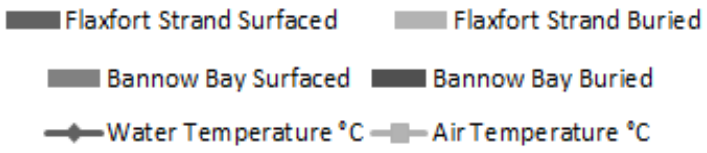
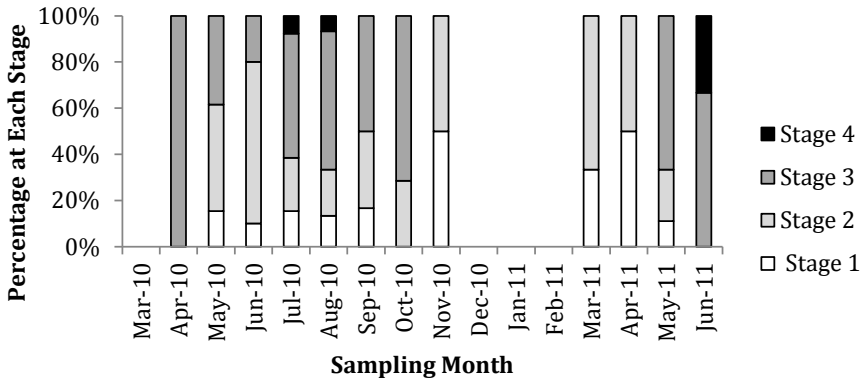


Figure 2: Prevalence of disseminated neoplasia at each site, with additional water and air temperature, for the duration of the study.

a.



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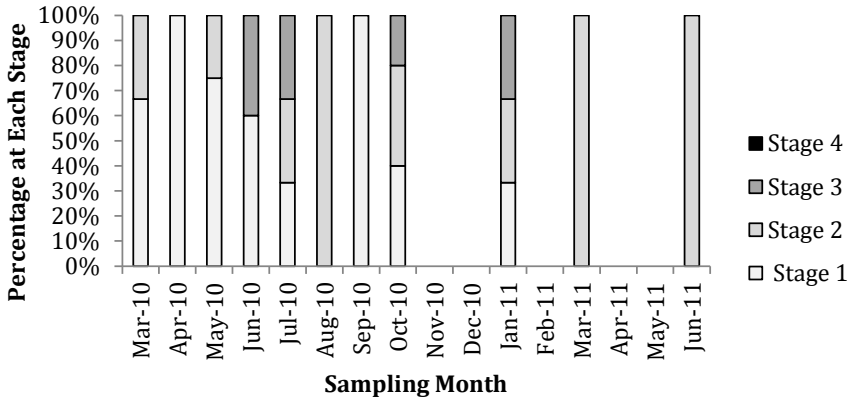
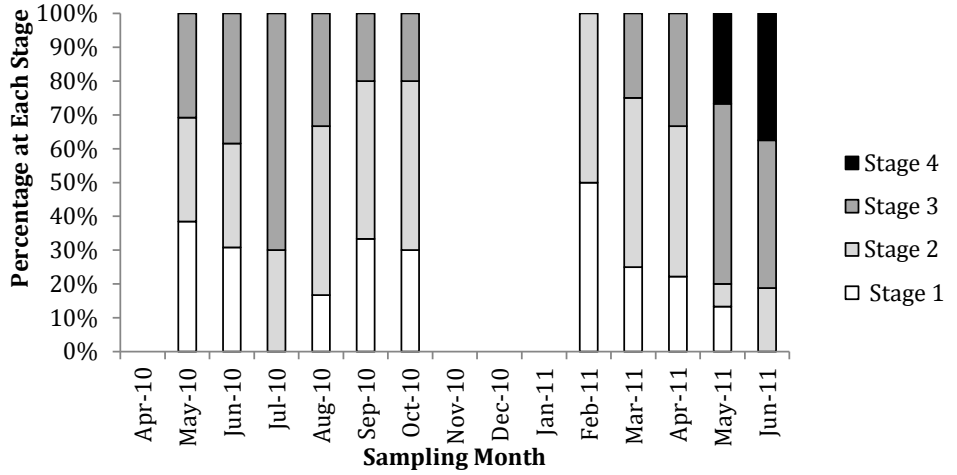


Figure 3: Intensity of neoplasia per month at a. surfaced and b. buried cockles at Bannow Bay.

a.



b.

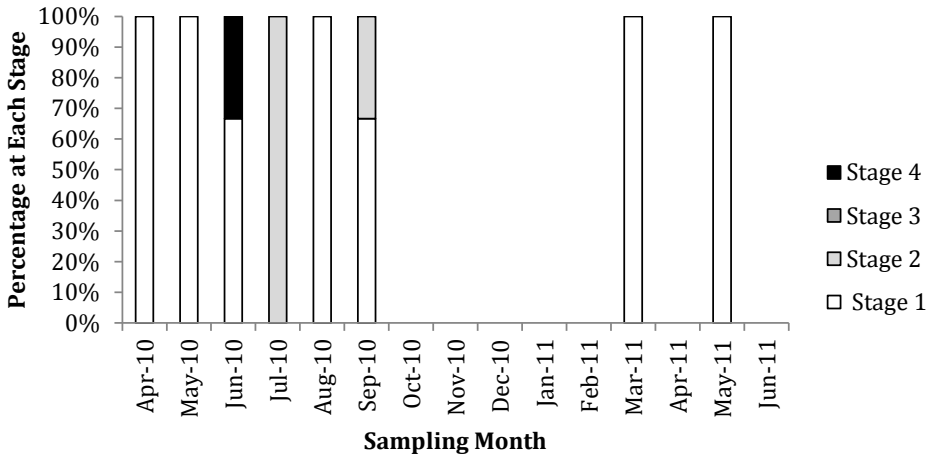


Figure 4: Intensity of disseminated neoplasia per month over the study period in a. surfaced cockles and b. buried cockles at Flaxfort Strand.

5.3.2 *Comparison of parasite species between surfaced and buried cockles at each site*

In relation to trematode species present there was a higher prevalence of only one trematode - *Labratrema minimus* sporocysts in buried (BB s 1.2 ± 2.17 %: b 1.6 ± 2.14 %, FF s 1.0 ± 1.92 %: b 1.1 ± 2.0 %) cockles from both sites. But the intensity of infection was greater in surfaced cockles at both sites (BB s 71.0 ± 126.57: b 50.2 ± 86.38 sporocysts, FF s 48.0 ± 93.07: b 30.0 ± 66.86 sporocysts).

Sporocysts in combination with metacercariae were more prevalent in surfaced cockles at each site (BB s 3.9 ± 3.89 %: b 1.8 ± 2.49 %, FF s 2.6 ± 3.51 %: b 1.8 ± 2.12 %); sporocysts in combination with metacercariae thought to be *Meiogymnophallus minutus* was the most common arrangement and once with *M. minutus* and *Himasthla interrupta*. The most common species of metacercariae was *Meiogymnophallus minutus* (BB s 59.1 ± 12.72 %: b 47.2 ± 21.28 %, FF s 68.8 ± 27.87 %: 64.6 ± 20.16 %), followed by *Himasthla interrupta* (BB s 12.3 ± 12.15 %: b 11.1 ± 13.81 %, FF s 3.6 ± 4.68 %: 2.8 ± 2.75 %), then *Gymnophallus choledochus* but that was not present in all categories of animals (surfaced/buried at both sites). *Nematopsis* sp. was prevalent at each of the sites (BB s 83.2 ± 16.11 %: 75.0 ± 27.96 %, FF s 85.8 ± 15.75 %: 89.7 ± 13.68 %). Its prevalence was greater in surfaced cockles from Bannow Bay, and its intensity was significantly greater at this site but not in Flaxfort Strand. *Rynchodida* and *Trichodinia*-like ciliates had higher intensities in buried cockles at both sites (Table 4). The prevalence of both ciliate species was greater in buried cockles at Flaxfort Strand, whereas equal prevalences of *Trichodinia*-like ciliates in surfaced and buried cockles was seen at Bannow Bay. *Rynchodida*-like cilates were more prevalent in surfaced cockles in Bannow Bay (Table 3).

The turbellarian *Paravortex cardii* was identified in surfaced and buried cockles from Flaxfort Strand, with a higher prevalence and intensity in buried cockles but in surfaced animals from Bannow Bay only. An unidentified cestode species was distinguished in surfaced cockles from Bannow Bay only too, and it was of a low prevalence (Table 3). At Bannow Bay and Flaxfort strand, a fungal infection was clear in cockles from each of the subsamples, it was more prevalent in surfaced animals at both sites. *Rickettsia*-like organisms were more common in buried cockles at both sites, unidentified bacteria 1 were more common in surfaced cockles at both sites but marginally so in Bannow Bay. Surfaced cockles in Bannow Bay only had gill cysts, while unidentified bacteria 2 were found in surfaced cockles at Flaxfort Strand (Table 4).

Table 3: Mean prevalence ± s.d. of each parasite over the course of the study. *Indicates significant difference.

Parasite	**Bannow Bay**		**Flaxfort Strand**	
	Surfaced	Buried	Surfaced	Buried
Labratrema minumus	1.2 ± 2.17	1.6 ± 2.14	1.0 ± 1.92	1.1 ± 2.00
L. minimus* and *Meiogymnophallus minutus	3.9 ± 3.89	1.8 ± 2.49	2.6 ± 3.51	1.8 ± 2.12
M. minutus	59.1 ± 12.72	47.2 ± 21.28	68.8 ± 27.87	64.6 ± 20.16
Himasthla interrupa	12.3 ± 12.15	11.1 ± 13.81	3.6 ± 4.68	2.8 ± 2.75
Gymnophallus choledochus	0	0.6 ± 1.33	0.2 ± 0.85	0
***Nematopsis* sp. oocysts**	83.2 ± 16.11	75.0 ± 27.96	85.8 ± 15.75	89.7 ± 13.68
***Trichodinia*-like ciliates**	4.5 ± 9.11	4.5 ± 5.59	10.6 ± 9.36	12.7 ± 13.91
***Rynchodida*-like ciliates**	3.3 ± 4.14	2.7 ± 2.58	6.9 ± 4.57	7.1 ± 5.40
Paravortex cardii	0.2 ± 0.88	0	0.2 ± 0.85	0.6 ± 1.37
Cestode	0.2 ± 0.88	0	0	0
Fungus	2.1 ± 3.09	0.4 ± 1.72	6.0 ± 5.86	2.8 ± 3.96
***Rickettsia*-like organisms**	1.2 ± 2.10	2.0 ± 3.50	0.2 ± 0.88	0.88 ± 1.51
Bacteria 1	2.4 ± 3.55	2.4 ± 3.45	3.1 ± 3.33	1.9 ± 2.36
Bacteria 2	0	0	0.7 ± 1.40	0
Gill cyst	0.2 ± 0.88	0	0	0

Table 4: Mean intensity ± s.d. of each parasite over the course of the study. *Indicates significant difference.

	Bannow Bay		**Flaxfort Strand**	
Parasite	Surfaced	Buried	Surfaced	Buried
Labratrema minimus	71.0 ± 126.57	50.2 ± 86.38	48.0 ± 93.07	30.0 ± 66.86
L. minimus* and *M. minutus	93.3 ± 144.7	119.2 ± 212.29	75.3 ± 111.11	48.0 ± 61.84
Meiogymnophallus minutus	33.2 ± 15.69	23.3 ± 12.46	27.7 ± 12.80	28.1 ± 11.31
Himasthla interrupa	0.8 ± 0.74	0.94 ± 0.84	0.6 ± 0.57	0.6 ± 0.56
Gymnophallus choledochus	0	0.1 ± 0.25	0.1 ± 0.25	0
***Nematopsis* sp. oocysts**	***46.2 ± 24.25**	**26.8 ± 18.57**	19.9 ± 8.33	23.3 ± 12.34
***Trichodinia*-like ciliates**	0.6 ± 1.20	0.7 ± 1.02	1.03 ± 0.78	1.3 ± 1.14
***Rynchodida*-like ciliates**	17.8 ± 37.38	26.3 ± 40.42	22.4 ± 19.59	56.9 ± 132.20
Paravortex cardii	0.1 ± 0.26	0	0.1 ± 0.26	0.3 ± 0.59
Cestode	0.1 ± 0.26	0	0	0

5.3.3 *Morphological changes*

Table 5: Mean prevalence ± s.d. of disseminated neoplasia and morphological changes observed over the course of the study. *Indicates significant difference.

	Bannow Bay		**Flaxfort Strand**	
	Surfaced	Buried	Surfaced	Buried
Neoplasia	**21.8 ± 16.25***	**6.4 ± 6.12**	**27.1 ± 20.18***	**4.2 ± 5.69**
Haemocyte Infiltration	0.5 ± 1.25	0.4 ± 1.16	0	0
Tissue Damage	1.2 ± 2.80	2.2 ± 4.83	1.4 ± 2.5	0

Two morphological changes were apparent, haemocyte infiltration and tissue damage (such as broken digestive tubules). Haemocyte infiltration was confined to Bannow Bay only and was more prevalent in surfaced cockles (Table 5). Tissue damage (e.g. broken gills) was found in surfaced cockles from Flaxfort Strand only, but it was more prevalent in buried cockles at Bannow Bay.

5.3.4 *Sex and parasites and pathological conditions*

There was no significant difference in the numbers of males and females with trematodes, neoplasia, or *Nematopsis* sp. among surfaced and buried cockles at either site.

5.3.5 *Combination of digenea and disseminated neoplasia*

Neoplasia and trematode infections (all reproductive stages) occurred in significantly greater numbers among surfaced rather than buried cockles at both sites (χ^2 test).

5.3.6 *Temperature and pathological conditions or parasites*

Temperature was correlated with prevalence of trematode sporocyst and trematode sporocysts in combination with trematode metacecariae in surfaced cockles in Flaxfort Strand only (Pearson product moment correlation, P= 0.05). The prevalence of disseminated neoplasia was correlated positively with air and water temperature in surfaced cockles in Bannow Bay and Flaxfort Strand (Pearson product moment correlation, P= 0.001).

5.3.7 *Comparison of cockles across all variables - Non-metric multidimensional scaling and analysis of similarity (ANOSIM)*

(a.) Bannow Bay

Non-metric multidimensional scaling ordination (NMDS) of the samples had a 2d stress of 0.12 and a variable stress of 0 was returned. Lower stress levels in the model suggest (below 0.2) that the model was not under undue stress to represent the data accurately in the ordination (Figure 5).

There was a great deal of similarity in the distribution of surfaced and buried cockles at Bannow Bay (Figure 5a), however there were some albeit non-significant differences apparent (Global R = 0.0.004 P = 0.146). The majority of buried cockles were aggregated together in the centre of the ordination and comparison against the variable ordination showed that no one parasite was characteristic of this group. In contrast surfaced cockles had a wide distribution

in the ordination, suggesting that a wider range of parasites especially digenea, *Nematopsis* sp. oocysts and *Trichodinia*-like ciliates were more prevalent and intense in this group.

There was no significant difference in the distribution of the sexes in the ordination, but trends were apparent (Global R = 0.043 P = 0.01) (Figure 5b). Males and indeterminate sex were characterised more so by digenea, *Nematopsis* sp. oocysts and *Rynchodida*-like ciliates, while females had a greater prevalence of *Trichodinia*-like ciliates.

Although not significantly different, the various stages of gonadal maturity did show different ordinations and associations with different parasites (ANOSIM Global R = 0.03 P = 0.07). Cockles that were spawning/partially spent or with no gonadal development were related to the greatest number of parasites and were associated with neoplasia therefore neoplasia may have been impacting on gonadal development. Developing cockles and spent individuals were very clustered in their distribution, and were also distributed away from most parasites (Figure 5c).

Seasonality was shown in the ordinations, and summer months were associated more so with disseminated neoplasia. July, August 2010, and June 2011 had wide dispersals in the ordination indicating that a wide number of parasites were prevalent in these months (Figure 5d). There was no significant difference between months though (ANOSIM Global R = 0.094 P = 0.01).

Trends were clear in parasite prevalence and intensity when cockles were compared across number of growth rings but they proved non-signficant (ANOSIM Global R = -0.003 P = 0.57). Cockles with one or two growth rings had a very aggregated distribution and were positioned in the centre of the ordination away from the ordination of parasites. Cockles with three growth rings had a much greater dispersal and therefore had higher prevalence and intensity of multiple parasites, and neoplasia in particular (Figure 5e).

NMS Key for both figures:

(a.) Position: **1** Bannow Bay surfaced, **2** Bannow Bay buried, **3** Flaxfort Strand surfaced, **4** Flaxfort Strand buried.

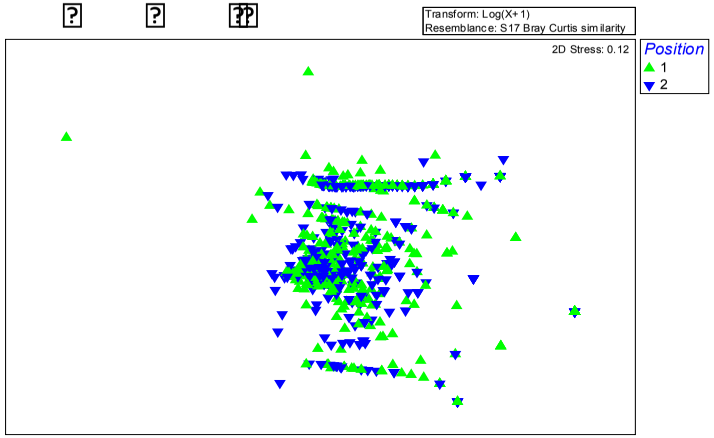
(b.) Sex: **0** indeterminate, **1** male, **2** female, **3** hermaphrodite.

(c.) Gonadal maturity: **0** no gonads, **1** early developing, **2** late developing, **3** ripe, **4** spawning/partially spent, **5** spent.

(d.) Month: **4** April 2010, **5** May 2010, **6** June 2010, **7** July 2010, **8** August 2010, **9** September 2010, **10** October 2010, **11** November 2010, 12 December 2010, **13** January 2011, **14** February 2011, **15** March 2011, **16** April 2011, **17** May 2011, **18** June 2011.

(e.) Number of growth rings: **1** = one growth ring, **2** = two growth rings, **3** = three growth rings, **4** = four growth rings, **5** = five growth rings, **6** = six growth rings, **7** = seven growth rings, **8** = eight growth rings.

(a.)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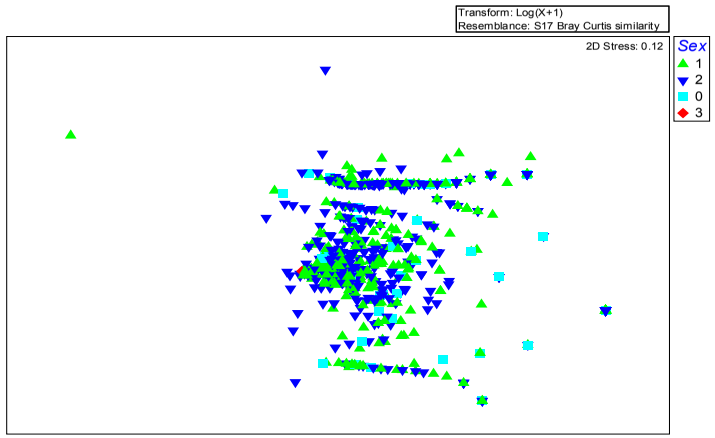
2

(b.)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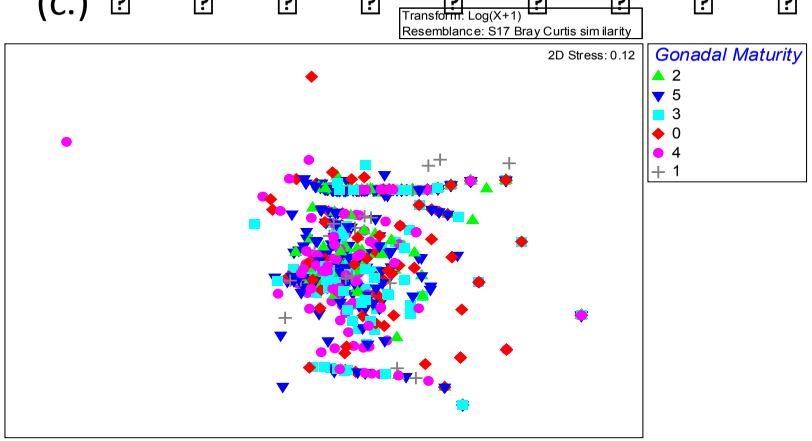
(c.)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Gonadal Maturity
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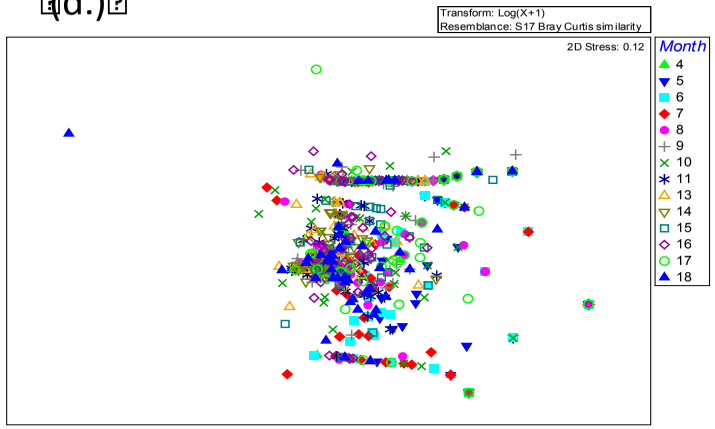
(d.)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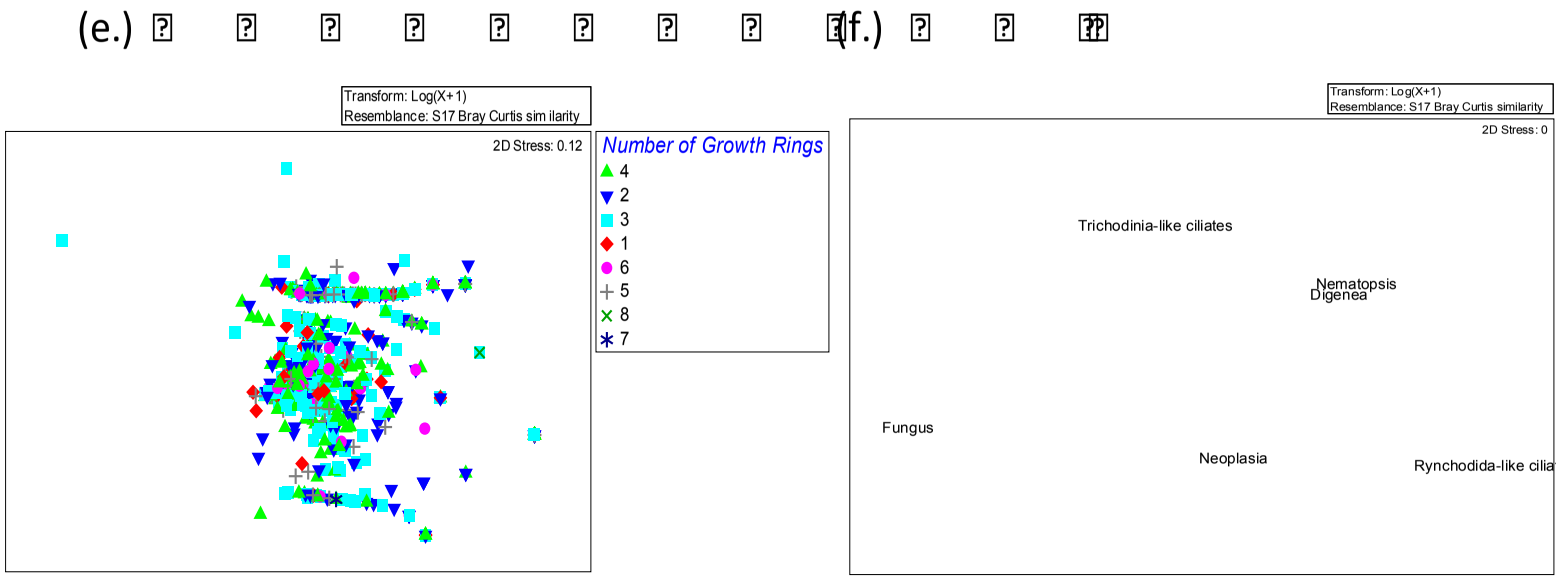


Figure 5: Non-metric multidimensional scaling ordination of (a.) position, (b.) sex, (c.) month, (d.) gonadal maturity, and (e.) number of growth rings at Bannow Bay.

(b.) Flaxfort Strand

The non-metric multidimensional scaling ordination (NMDS) of the samples had a 2d stress of 0.12 and the variable stress was 0.02. As previously mentioned the lower the stress level, especially if it is below 0.2 indicates that the model was not under undue stress to represent the data accurately in the ordination.

There was some variation apparent in the ordination of surfaced (code = 3) and buried cockles (code = 4) in Flaxfort Strand. However they were not significantly different across all measured characters (ANOSIM Global R = 0.014 P = 0.01). Digenea, *Nematopsis* sp. and neoplasia and a greater range of parasites were more prevalent and abundant in surfaced cockles. Buried cockles were distributed away from the ordination of most parasites; therefore parasites such as ciliates, neoplasia, and trematodes were not characteristic of these buried cockles.

Males and females did show some overlap in their ordination, however differences were apparent but they did not prove significant (ANOSIM Global R 0.008 P = 0.191). *Rynchodida*-like ciliates were more prevalent in males, while digenea and nematopsis may be more common to females. Fungal growth was more characteristic of cockles of indeterminate sex.

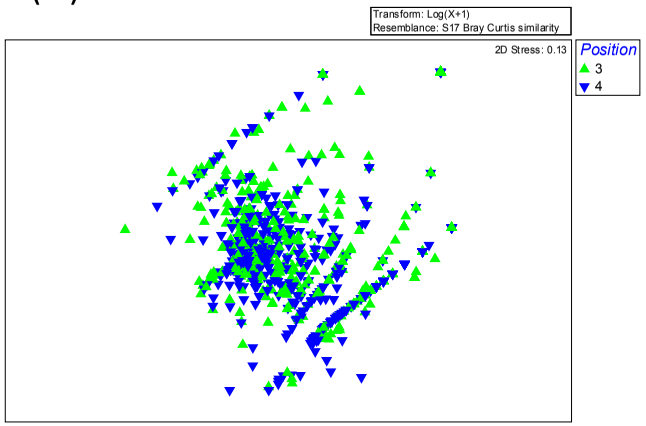
There was no significant difference between cockles with varying levels of gonadal maturity, but trends were apparent (ANOSIM Global R = 0.02 P = 0.04). Cockles with no gonads, those that were spawning/partially spent and spent had a great deal of spread in their ordination. Therefore they had a greater prevalence and abundance of a wide range of parasites such as neoplasia, digenea, and ciliates. In contrast early and late developing cockles were clustered together away from those with parasites.

The months included in the study did show variation in the ordination, but it did not prove to be significant (Global R = 0.06 P 0.01). July 2010 showed a wide distribution, indicating that several parasites such as digenea, ciliates, and neoplasia were prevalent in this month. May and June 2011 showed similar distributions and were linked to digenea, neoplasia and *Rynchodida*-like ciliates.

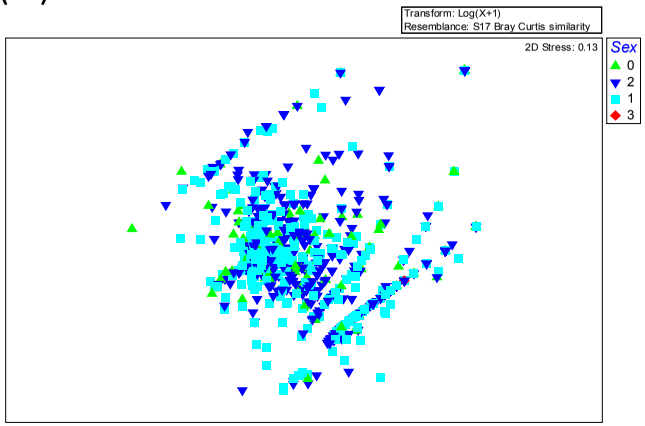
The strongest (non significant) trend was clear in terms of number of growth rings per cockle (ANOSIM Global R = 0.38 P = 0.08). From the sample and variable ordination it is clear that the range, prevalence and intensity of parasites

increases with the number of growth rings. As the number of growth rings increases the distribution of cockles become closer and closer to the ordination of the parasites and neoplasia. Cockles with three growth rings are the most widely distributed and characterised by a greater range of parasites, but from four growth rings onwards cockles again become more clustered and not affected by so many parasites and neoplasia indicating that 3 + age group is a critical threshold for disease development and subsequent mortality.

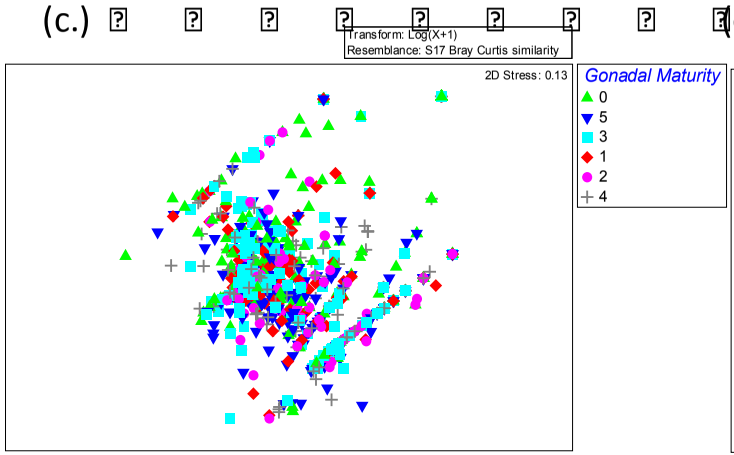
(a.)


Transform: Log(X+1)
Resemblance: S17 Bray Curtis similarity
2D Stress: 0.13
Position
3
4

(b.)


Transform: Log(X+1)
Resemblance: S17 Bray Curtis similarity
2D Stress: 0.13
Sex
0
2
1
3

(c.)


Transform: Log(X+1)
Resemblance: S17 Bray Curtis similarity
2D Stress: 0.13
Gonadal Maturity
0
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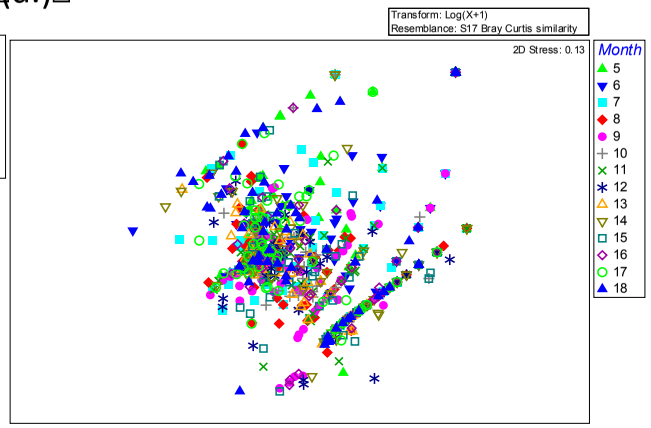
(d.)


Transform: Log(X+1)
Resemblance: S17 Bray Curtis similarity
2D Stress: 0.13
Month
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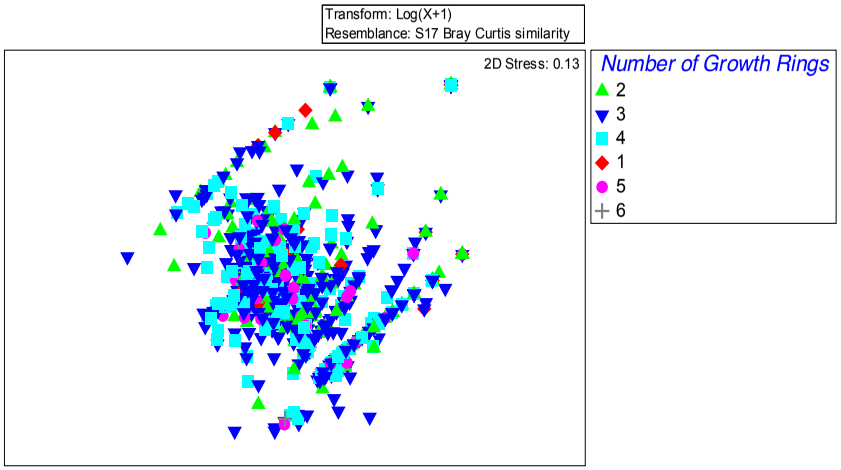
(e.)



(f.)

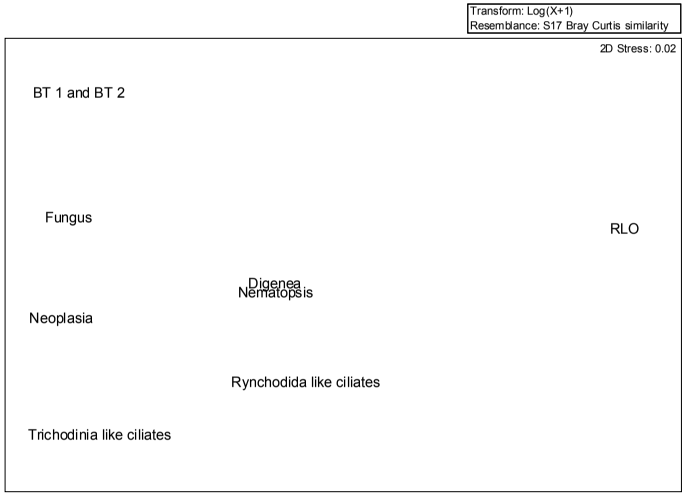


Figure 6: Non-metric multidimensional scaling plots of (a.) position, (b.) sex, (c.) gonadal maturity, (d.) month, (e.) number of growth rings, and (f.) variable ordination for Flaxfort Strand.

5.4 DISCUSSION

The present study of the parasites and pathological conditions of an ecosystem engineer, the cockle, emphasised the influence of local conditions on cockle surfacing and mortalities at two separate sites. The combination of infection with a wide range of parasites, in addition to season and age, appears to influence surfacing and mortality in cockles from Bannow Bay. In contrast, surfaced cockles from Flaxfort Strand had a greater prevalence of neoplasia and trematodes. Three years appeared to be a critical age to attain a threshold level of parasites after which mortality may occur. It appears that older surviving cockles at this site are less susceptible to disease. Inherent site characteristics, in addition to spatial variation in cockle populations and environmental differences, may be expressed in the diversity and intensity of parasites and pathological conditions. Differences in sites that are separated by 100 km or more are thought to be due to largely environmental factors and those separated by a great distance are unlikely to have the same conditions resulting in dissimilar communities (Wilson *et al*., 2011).

Attempts were made using multivariate analysis to explain the occurrences of surfaced cockles, exploratory NMDS ordinations showed low stress values indicating that the ordinations were a good representative of the datasets (Clarke and Gorely, 2006). Subsequent statistical analysis highlighted the difficulty in using multivariate analysis in histological studies due to sample size, which may be why it is not often used for parasitological studies. Low values were returned for the Global R statistic in ANOSIM indicating no difference (less than 0.2), but the large number of samples created significant P values, as it is a function of the large sample size used (n = 706, n = 782 and n = 1488). Global R is the measure of absolute differences between groups and the greater the value the better (Clarke and Gorely, 2006). A large number of replicates and permutations were performed so the test is believed to be reliable therefore Global R values returned were deemed reliable (Clarke and Warwick, 2001). Univariate statistics utilised in the first two sections and patterns identified in the exploratory NMDS and ANOSIM analysis highlighted several trends in the data however. Despite the fallibility of multivariate statistics in studies such as this, their use does allow for the analysis of a large dataset with many factors, and can emphasise trends or patterns in the data, which was the case here.

The presence of trematodes even in low numbers at both sites is indicative that other hosts in its life history are present at both sites, or as its final hosts are water birds,

pay visits, however ephemeral, to these sites (Marcogliese and Cone, 1997). The distribution of the final host especially, if it is a bird species, is thought to be key to transmission of parasites to hosts (Hechinger and Lafferty, 2005). The high prevalence of *Meiogymnophallus minutus* on Irish shores has been remarked upon previously and it may be due to high densities of the second intermediate host the common cockle (Fermer, 2010). Higher prevalence and intensity at Bannow Bay of *Himasthla interrupta* could be related to the abundance of its first intermediate host, *Hydrobia ulvae* that were often found in close association with cockles, and during the summer months they were recovered from within the cockle valves of still living surfaced animals.

Irrespective of site, position on or in the sediment *Nematopsis* sp. oocysts were extremely prevalent and despite often-high intensities being observed, no host response was evident. The prevalence and intensity of *Nematopsis* sp. has increased markedly since a previous study in Ireland performed in Flaxfort Strand (Morgan, 2009). *Nematopsis* sp. could reach up to 100 % prevalence per month and 76 % prevelence has been recorded previously in cockles from Galicia northwest Spain (Carballal *et al*., 2001) and 82 % in cockles experiencing mortalities from southern Portugal (Azevedo and Cachola, 1992). The rapid increase in prevalence and intensity of this gregarine, may be reflective of the alterations in population densities and community structure (within the cockle and the ecosystem at large), that climate variability, such as seen here, with the anomalous extremely cold winter, may bring about (Brooker *et al*., 2007). In a concurrent study, a health screen of *Mytilus edulis* was performed at both sites, although *Nematopsis* sp. was identified in mussels it was at very low levels (Chapter 6). Though *Nematopsis* sp. has been found in a range of bivalves at these sites, due to the high densities of cockles they might be preferentially parasitising this bivalve over others. Specialisation on a particular host has the incentive that parasites can generate coping mechanisms to deal with host response to avoid detection (Combes, 1997).

Reports of mortalities or mass surfacings of cockles have come from several locations across Europe – various aetiologies have been proposed, each differing per site (e.g. trematodes, haplosporidia) (Anonymous 2011; Carballal *et al*., 2001). Comparing the prevalence and intensities of parasites between surfaced and buried cockles at both sites showed that the source of surfacing was not duplicated at both sites. Disseminated neoplasia and trematodes co-occurred significantly more often in

surfaced rather than buried cockles at both sites, but the influence of other parasites was apparent especially at Bannow Bay and the influence of season was also highlighted by NMDS ordination. Surfaced cockles from Bannow Bay had, in general, higher prevalence and intensities of all parasites identified, whereas this was not the case at Flaxfort Strand. In animals affected by multiple parasite species, it is thought that the relationship between these parasites will be manifested in the severity of the disease outcome as well as host fitness (Pedersen and Fenton, 2006). High parasite burden from multiple sources, in addition to disseminated neoplasia, may have been the basis of cockle mortalities at this site. In particular as neoplastic cells lose the ability to phagocytose, depriving the cockle of an immune response this maybe a significant factor impacting on host – parasite interactions (Barber, 2004; Delaporte *et al*., 2008; Díaz *et al*., 2011). The influence of season at Bannow Bay was emphasized in analysis also as being a significant component as cockles are unable to survive extremes of temperature (Thieltges, 2006) and thermal stress has been emphasised as a mechanism by which the effects of parasites are exacerbated (Shim *et al*., 2013). This was particularly significant over the study period due to the extremes of temperature observed.

In general, hosts that are greater in size may be older; therefore they will have had a longer time period potentially in contact with infective stages (Thieltges, 2008). Cockles are known to accrue large numbers of trematodes, metacercariae in particular, without mortality (Wegeberg and Jensen, 1999). Surfaced cockles at both sites were older, larger (length, width, height, whole weight, tissue weight, shell weight) and had a lower condition index (proxy for ill-health) than buried cockles (Morgan unpublished data) (Orban *et al*., 2002). An age threshold was emphasized by NMDS ordination at both sites, and cockles with more age rings had a greater range, prevalence, and intensity of parasites. Older cockles would have had a longer time period to accumulate parasites, and larger animals may have a greater pumping rate so could have ingested more parasites via their inhalant siphons (but smaller animals have greater pumping rates per gram body weight) (Nikolaev *et al*., 2006). The highest prevalence of neoplasia was in cockles with three growth and the latter stages of the disease were more common in this age class. There may be a threshold level determined by temporal exposure in the case of parasite diversity and intensity per age. Neoplasia was first identified in cockles in Ireland in 1982 and while it is of unknown origin, a viral aetiology has repeatedly been hypothesised (Romalde *et al*.,

2007). Perhaps following 30 years of infection a proportion of cockles are becoming resistant to its vector. Cockles are *r*-selected relatively short-lived species; therefore there has been ample time for multiple generations of resistant animals to emerge.

In the host-parasite system, there may be a persistence-type threshold, which means a minimum number of parasites are needed to maintain the parasite (O"Grady *et al*., 2013). Similarly this threshold once exceeded may create a significant change in the host-parasite relationship and then mortalities may occur in the population (O"Grady *et al*., 2013). In the case of the cockle, the threshold of parasite intensity is reached and exceeded at age three, which in combination with high prevalence of neoplasia and the high intensity of the latter stages of the disease in this age class may result in surfacing and subsequent death.

The present study identified a range of parasites and one pathological condition in the common cockle. The study emphasised the influence of local, site related drivers of disease development and subsequent mortality events. In the case of Bannow Bay the combination of high parasite diversity, present in individual animals, along with high intensities induced mortalities in older animals, and a seasonal influence on mortality events was also a significant component. At Flaxfort Strand neoplasia in unison with digenean trematode infections was the basis of surfacing events. The use of multivariate statistics allowed for the comparison of cockles across all measured characters together. Surfacing is undoubtedly a complex phenomenon that is influenced by parasite species, prevalence, intensity, and the cockle physiology and is much subjected to local stressors both endogenous and exogenous to the individual.

5.5 ACKNOWLEDGEMENTS

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CHAPTER 6:

Section 1: Must parasitism impact on an individual? Assessing the impact of parasites on mussel health.

Section 2: Parasite diversity across all taxa.

6.1 ABSTRACT

Mytilus edulis is a commercially and ecologically important marine bivalve worldwide. It may form biogenic reefs and comprise a large portion of the intertidal biomass. The health status of *M. edulis* was examined at two sites in Ireland, 215 km apart and assessed across a range of parameters: site, temperature, month, sex, and stage of gonadal development. The questions were posed "what is a normal or acceptable level of parasitism in a population?" and "does parasitism always result in a negative impact on the health of the individual?" A range of parasites and pathologies were identified: trematode metacercariae were the most commonly encountered form of parasite at both sites and three distinct species were recorded. The parasite species recorded were specific to both sites. Despite these parasites and morphological changes, mussels at both sites were robust, reproduced normally and no evidence of mortalities that might be above normal levels was observed. Interestingly, despite having significantly greater prevalence and intensity of parasites, mussels from Flaxfort Strand were significantly greater in length and weight that those from Bannow Bay. This may show that parasitism does not equate to automatic ill health. The results reinforce the necessity for threshold levels of parasites to be present in the individual to induce negative impacts. External stressors will in turn impact upon these threshold levels that influence the animals at an individual, population, and community level.

6.2 INTRODUCTION

The world's population continues to increase and is expected to reach 9.3 billion by 2050 (Akaishi *et al*., 2007) and greater demands are put on the marine environment with 60 % of the world's population within 100 km of the coast (Vitousek *et al*., 1997). In estuarine areas, many birds, fishes, and bivalves with either a commercial or ecological significance, or both, exploit these areas as nursery and feeding grounds (Akaishi *et al*., 2007). These include the blue mussel *Mytilus edulis*, which is commercially important, globally accounting for 8 % of world mussel production by species (Beaumont *et al*., 2007; Morley, 2010). *Mytilus edulis* comprises large amounts of intertidal biomass, they are a food source for a range of birds, they themselves also offer habitat as a "biogenic reef" for a range of invertebrates and play a role in nutrient cycling (Dankers and Zuidema, 1995).

Several pathogens i.e. any bacterium, virus or other agent such as microorganisms that causes disease, have been identified in mussels worldwide, which may impact on condition, meat quality, and provision of mussel seed, however some believe investigations into mussel pathogens is still lacking (da Silva *et al*., 2002). These include digenean trematodes, the copepod *Mytilicola intestinalis*, microsporidia (e.g. *Steinhausia mytilovum*), protozoans, and Picorniviridae like viruses (www1; de Montaudouin *et al*., 2000; Comtet *et al*., 2004; Parry and Pipe, 2004; Thieltges *et al*., 2006; Prinz *et al*., 2010). Previous studies performed by Prinz *et al*. (2009 & 2010) in Ireland have shown that *M. edulis* acts as a second intermediate host for the digenean trematode *Echinostephilla patellae* on rocky shores. Additionally, studies from the United Kingdom have shown mussels play host to various trematodes, copepods, ciliates, and *Rickettsia*-like organisms (Bignell *et al*., 2008; Bignell *et al*., 2011) but few studies have focused on the health status of *Mytilus edulis* in Ireland.

In any free-living population it is inevitable that some individuals will be parasitised (Poulin, 1999; Bordes and Morand, 2009). However the question must be raised, is there an acceptable level of parasitism for the individual or the population, beyond which the population is susceptible to mortalities? Automatically equating parasitism to ill health is a very broad-spectrum assumption, and due to the prevalence and ubiquity of parasites (macro- and micro-) this would mean that the vast majority of organisms worldwide are unhealthy or at least sub optimal. Admittedly the host-parasite relationship is a special case of a symbiotic relationship, with the parasite exerting some harm on the host (Thieltges *et al*., 2012) but a well-adapted parasite is one, that is intermediately pathogenic or non-pathogenic (Combes, 1997).

Therefore the parasite fauna of *Mytilus edulis* was of interest, as despite it being parasitised, and at the same site where high mortalities have been observed in another bivalve *Cerastoderma edule* (e.g. Fermer 2010, Morgan *et al*., 2012) there have been no reports of mass mortalities in this species. The parasite species, plus their prevalence and intensity was of interest as to determine a "normal" or acceptable level of parasitism.

Thresholds exist in the host parasite relationship; a minimum number of hosts are required, to allow for transmission to new hosts and to maintain the parasite in the environment (Lafferty and Kuris, 1999; Torchin *et al*., 2002). The rate of transmission to new hosts must exceed the rate of recovery or death of already infected individuals

(Lafferty and Kuris, 1999). Within a host too a threshold level of parasites is required, after which the host-parasite relationship is altered and beyond which mortalities can occur (O'Grady *et al*., 2013). Parasites, in particular helminths, can be plastic in their response to the host environment (Fenton and Rands, 2004) but the trade off theory states that the evolution of parasite virulence is hampered by the interactions of transmission rate, host recovery and parasite virulence itself (Coors and De Meeter, 2011). For a parasite to become pathogenic, and disease or mortalities to occur certain conditions are required. Disease has been defined as "any impairment that interferes with or modifies the performance of normal functions, including responses to environmental factors such as toxicants and climate, nutrition, infectious agents; inherent or congenital defects or any combination of these factors" (Hedrick, 1998). From this definition it is clear that disease and subsequent mortalities are a complex interaction between the host, environment, and parasite (Hedrick, 1998). The scales of ill effects that occur as a result of parasitism are dependent on how the host, parasite, and environment interact (Hedrick, 1998).

Metabolic age, as a measure of physical health can be assessed by the acquisition of lipofuscin granules (Riveros *et al*., 2002; Dimitriadis *et al*., 2004). Lipofuscin granules are a type of insoluble cellular biomarkers, which can reflect the sublethal effects of pollutants (and imply their presence in the environment) and cell damage (Riveros *et al*., 2002). Metal detoxification can be aided by lipofuscin granules (Riveros *et al*., 2002; Dimitriadis *et al*., 2004). It has been shown that *Mytilus edulis* had higher levels of lipofuscin granules in the presence of high pollutant concentrations (Dimitriadis *et al*., 2004). Therefore their presence or absence was noted in the present study – as both sites are SACs and SPAs, and therefore should be of good quality.

Two sites Bannow Bay and Flaxfort Strand, 215 km apart were chosen in the present study to assess parasite diversity and load at each site and to assess if site related differences exist. Both sites are classified as Special Areas of Conservation and Special Protected Areas under the EU Birds Directive and EU Habitat Directive with many bird species e.g. the oystercatcher acting as definitive hosts of trematodes which may have an intermediate stage in the mussel. Despite reported "unusual" mortalities in another bivalve *Cerastoderma edule* (e.g. Fermer 2010; Morgan *et al*., 2012; Chapter 5) at Flaxfort Strand and Bannow Bay no such observations have been made in *Mytilus edulis* in these regions. Mortalities may have been masked by bird and fish predation on

mussels as plaice (*Pleuronectes platessa*) and oystercatchers (*Haematopus ostralegus*) predate upon them (Hilgerloh, 1997). However at the same site, high numbers of surfaced cockles were present despite bird and fish predation (personal observation).

6.3 RESULTS

A total of 13 parasite species were identified during the course of the study these included trematode sporocysts, three species of trematode metacercariae, two species of copepod and a fungal growth. Seven morphological changes such as lipofuscin granules in the mantle and organs, granulomas and presence of eosinophilic cells in the digestive system, which may have been a reproductive stage of a parasite, were recorded also. All 13 parasites were not recorded at both sites and high prevalence and or intensity per month at one site did not correspond to the same phenomenon at the other; trematode sporocysts, *Trichodinia*-like ciliates, and cestodes were found exclusively at Flaxfort Strand however the morphological changes were common to both sites. From the results it is clear that there was a much higher prevalence of infection at Flaxfort Strand, but the intensity of parasites between sites was comparable. 57.5 % of mussels from Bannow Bay had no parasites in their histological sections, 32.0 % had single infections, 9.5 % had dual infections, and 1.4 % had three infections. In comparison 34.8 % of mussels from Flaxfort Strand had no parasites observed, 42.0 % were infected by one parasite taxa only, 19.7 % had dual infections, 3.1% had three parasites infecting them and only 0.44 % had four parasite taxa combined. Reproductive and size data referred to in this chapter were outlined previously in Chapter 4.

6.3.1 *Polymerase Chain Reaction*

No mussels examined from either site tested positive for the conditions screened for using PCR. All positive controls showed bands and there were no bands on the negative controls.

6.3.2 *Parasites*

Table 3: Mean prevalence ± s.d. of parasites identified at both sites over the study period. * Indicates significant difference.

	Bannow Bay (%)	**Flaxfort Strand (%)**
Bucephalid sporocysts	0	0.2 ± 0.85
Gymnophallus choledochus*	12.6 ± 8.90	36.2 ± 15.59
Himasthla interrupta*	5.6 ± 4.75	26.5 ± 17.16
Himasthla elongata*	0.9 ± 2.42	9.5 ± 11.17
***Nematopsis* sp. oocysts**	0.4 ± 1.16	2.0 ± 2.82
Mytilicola intestinalis	5.0 ± 6.44	8.9 ± 7.63
Copepod - unknown species	0.5 ± 1.29	0.2 ± 0.88
Fungus - unknown species	16.6 ± 11.31	16.8 ± 13.49
Ciliate unknown species	9.7 ± 7.09	7.6 ± 8.13
***Trichodinia*-like ciliates**	0	0.2 ± 0.92
Ancistrum mytili	2.1 ± 4.63	2.7 ± 2.63
Cestode	0	0.2 ± 0.85
***Rickettsia*-like organisms**	2.3 ± 3.14	1.4 ± 2.17

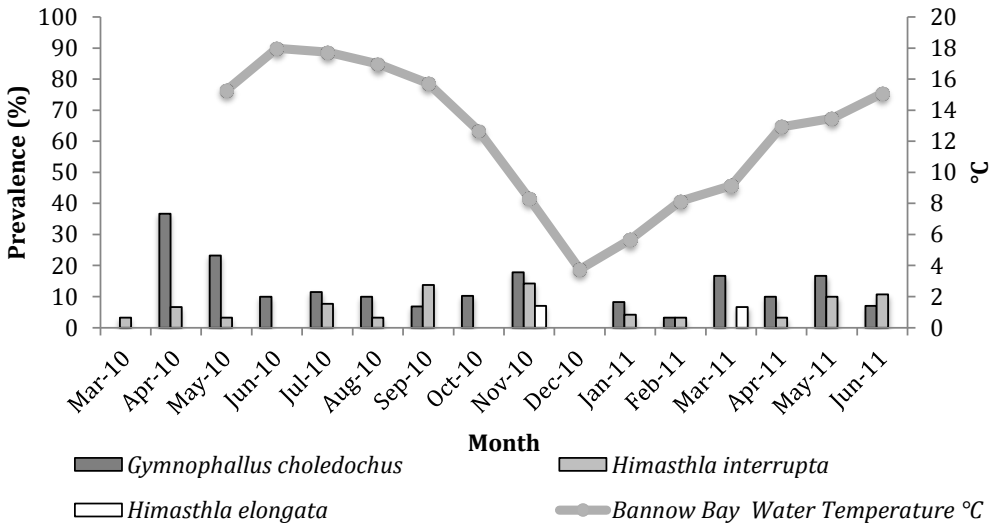
Bucephalid sporocysts were identified in one mussel from Flaxfort Strand in December 2010 only (Table 3). Trematode metacercariae of *Gymnophallus choledochus* were the most prevalent at both sites, and significantly more so at Flaxfort Strand (BB 12.6 ± 8.90 %: FF 36.2 ± 15.59 %). *Gymnophallus choledochus* also had a significantly higher mean intensity in Flaxfort Strand than in Bannow Bay (BB 1.2 ± 0.95 metacercariae per section: FF 2.2 ± 1.08 metacercariae per section). *Himasthla interrupta* had both a significantly higher mean prevalence and in Flaxfort Strand than Bannow Bay (prevalence BB 5.6 ± 4.75 %: 26.5 ± 17.16 %) and intensity (BB 1.2 ± 1.12 metacercariae per section: FF 2.6 ± 1.26 metacercariae per section). The mean prevalence and intensity of *Himasthla elongata* were significantly higher in Flaxfort Strand than Bannow Bay (prevalence BB 0.9 ± 2.42 %: FF 9.5 ± 11.17 %) (Figure 2).

A fungus like infection was apparent in mussels from both sites. It had a marginally higher though non-significant mean monthly prevalence in Flaxfort Strand than Bannow Bay and it correlated positively with temperature (BB 16.6 ± 11.31 %: FF 16.8 ± 13.49 %). There was a higher prevalence of the copepod *Mytilicola intestinalis* in Flaxfort Strand than Bannow Bay (BB 5.0 ± 6.44 %: FF 8.9 ± 7.63 %) but the intensity was comparable between sites (BB 0.8 ± 0.59 copepods per section: FF 0.9 ± 0.46 copepods per section). The unknown copepod species was more prevalent and intense at Bannow Bay (0.5 ± 1.29 %) than Flaxfort Strand (0.2 ± 0.88 %) but not significantly so. The gill ciliate *Ancistrum mytili* and ciliates of unknown species were identified in mussels from Bannow Bay and in addition *Trichodinia*-like ciliates were recorded in Flaxfort Strand only. Each was more prevalent with a higher intensity at Flaxfort Strand but not significantly so. There was a higher prevalence of *Nematopsis* sp. in Flaxfort Strand (2.0 ± 2.82 %) than at Bannow Bay (0.4 ± 1.16 %) but they did not differ significantly. *Nematopsis* sp. also had a higher mean intensity at Flaxfort Strand than Bannow Bay. *Rickettsia*-like organisms (RLOs) associated with gill filaments were seen at both sites, but at a higher prevalence in Bannow Bay than Flaxfort Strand. The intensity of RLOs was greater also in Bannow Bay. One cestode was found exclusively at Flaxfort Strand, and in February 2011 only.

Table 4: Mean intensity ± s.d. of each parasite identified from both sites, * indicates significant difference.

	Bannow Bay	**Flaxfort Strand**
Bucephalid sporocysts	0	46.6 ± 180.48
Gymnophallus choledochus*	1.2 ± 0.95	2.2 ± 1.08
Himasthla interrupta*	1.2 ± 1.12	2.6 ± 1.26
Himasthla elongata*	0.13 ± 0.35	2.3 ± 2.38
***Nematopsis* sp. oocysts**	0.2 ± 0.56	0.5 ± 0.72
Mytilicola intestinalis	0.8 ± 0.59	0.9 ± 0.46
Copepod - unknown species	0.1 ± 0.35	0.1 ± 0.25
Ciliate - unknown species	1.2 ± 0.61	7.6 ± 8.13
***Trichodinia*-like ciliates**	0	0.2 ± 0.93
Ancistrum mytili	0.3 ± 0.45	2.7 ± 2.63
Cestode	0	0.1 ± 0.26

(a.)



(b.)

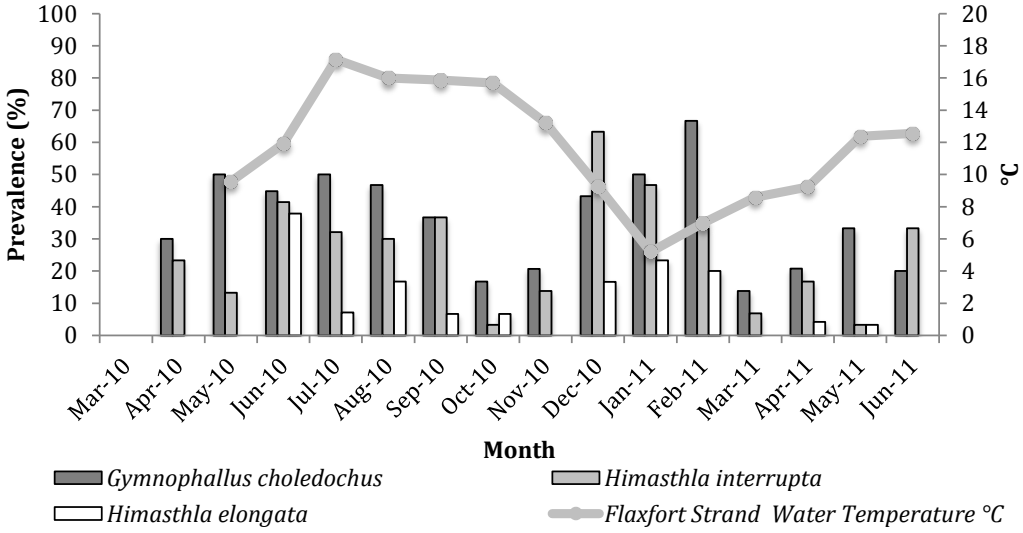
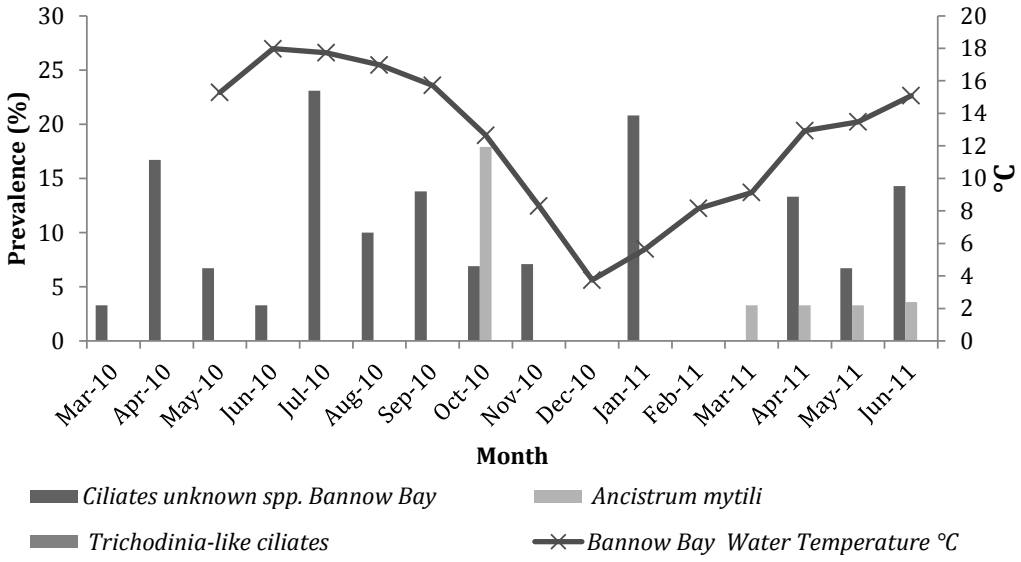


Figure 2: Prevalences of various trematode metacercariae recorded over the study period at (a.) Bannow Bay and (b.) Flaxfort Strand.

(a.)



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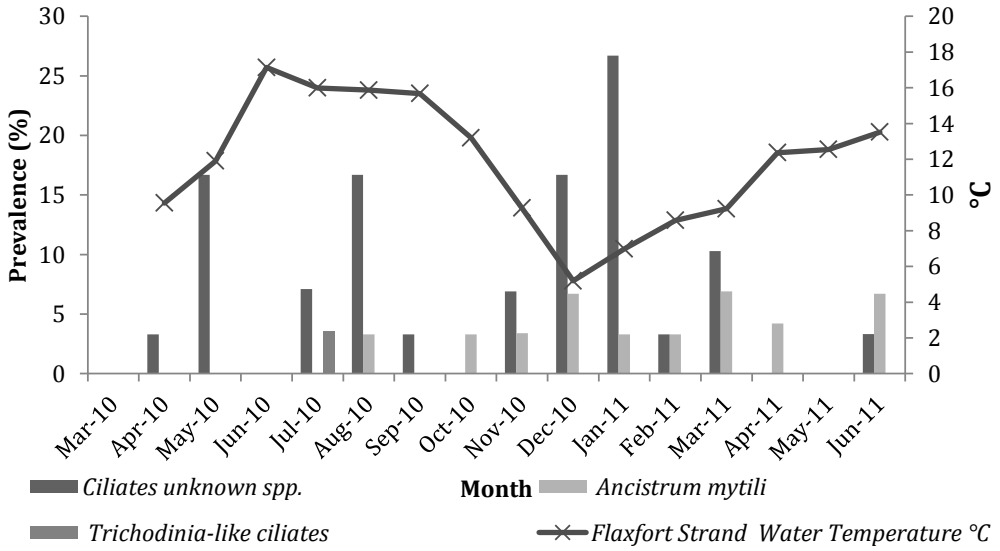


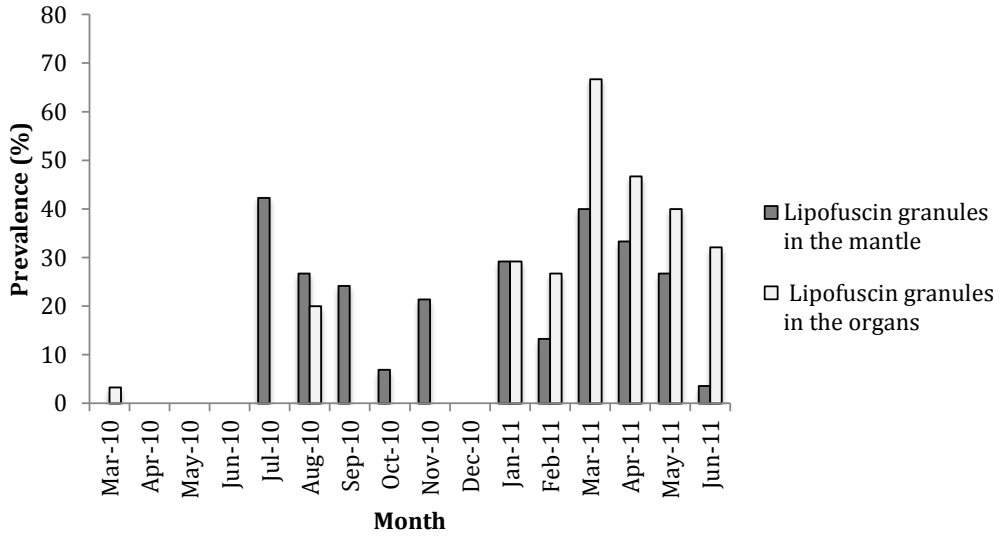
Figure 3: Prevalence's of various ciliate species identified in mussels at (a.) Bannow Bay and (b.) Flaxfort Strand during the course of the study.

6.3.3 *Morphological Changes*

Lipofuscin granules in the mantle (LM) and lipofuscin granules in the organs (LO) were the most common pathologies observed at both sites and they were more common in Flaxfort Strand than Bannow Bay (LM BB 17.8 ± 15.31 %: FF 27.7 ± 16.18 %, LO BB 17.6 % ± 21.65: FF 23.6 ± 17.92 %).

Eosinophilic cells (perhaps a reproductive stage of a parasite) were observed in the digestive tubules of mussels at both sites with a higher prevalence at Bannow Bay (BB 10.6 ± 22.67 %: FF 8.7 ± 8.43 %). Granulomas due to tissue injury or parasite encapsulation were also more common at Bannow Bay (6.0 ± 6.16 %) than Flaxfort Strand (5.0 ± 3.64 %) as were abnormalities in the gills (BB 1.8 ± 6.02 %: FF 0.9 ± 3.51 %) and haemocyte infiltration of the tissues (BB 1.4 ± 1.73 %: 1.3 ± 2.46 %). Digestive tubule and gonad damage or melanisation was more prevalent in Flaxfort Strand (1.9 ± 3.51 %) than Bannow Bay (1.1 ± 2.42 %). There was no significant difference in the prevalence of any aforementioned pathology between Bannow Bay and Flaxfort Strand.

(a.)



(b.)

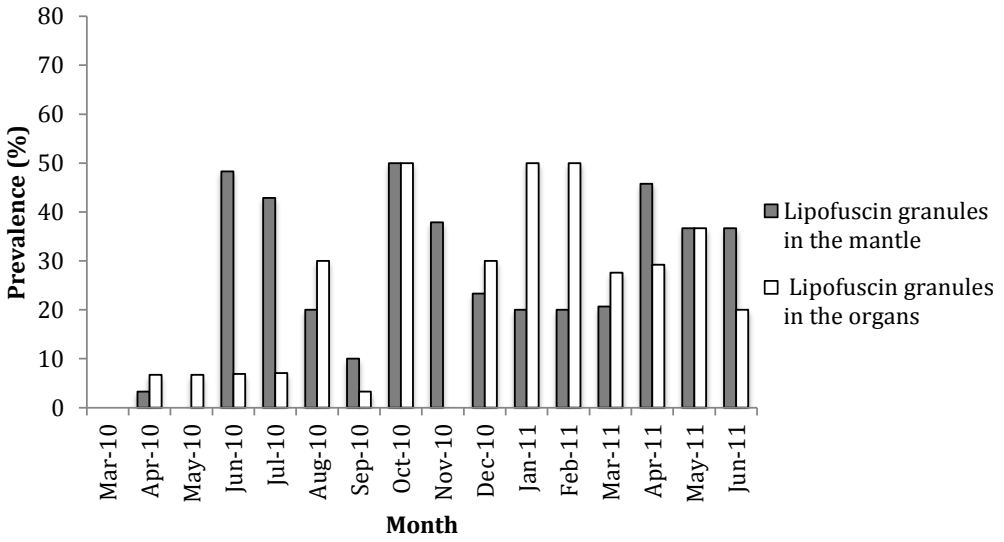


Figure 4: Prevalence of lipofuscin granules in the mantle and in the organs of mussels from (a.) Bannow Bay and (b.) Flaxfort Strand.

6.3.4 *Non-metric multidimensional scaling, one-way analysis of similarity (ANOSIM) and cluster analysis*

NMDS plots were created based on the factors site, month, sex, and stage of gonadal development (sample 2d stress = 0.11, variable 2d stress 0) (Figure 5 and 6).

The NMDS ordination showed a distinction between mussels from the different sites and this proved significant following ANOSIM analysis (Global R = 0.157, P 0.01). Digenea were more characteristic of mussels from Flaxfort Strand, and digenea were associated with fungal infection. Granulomas were more prevalent at Bannow Bay (Figure 5a).

There was no significant difference between male, female, and indeterminate mussels but they did have slightly different ordinations (ANOSIM Global R 0.016 P 0.05). Digenea and fungi were more common in female mussels, while males had a greater distribution across the ordination and therefore a wider range of parasites plus granuloma (Figure 5b).

Differences in month were apparent but proved to be non significant (ANOSIM Global R = 0.07). Mussels from June 2011 had a greater distribution in the ordination, and therefore digenea, fungus, ciliates, copepods, and granuloma were prevalent in these months. In contrast January 2011 had a very clustered distribution, and no one parasite was characteristic of this month (Figure 5c).

There was no significant difference in mussels with different stages of gonadal maturity but trends were apparent (ANOSIM Global R 0.01 P 0.111). Mussels that were classed as spawning/partially spent were more widely distributed in the ordination and had higher prevalence and intensity of parasites and granuloma. In contrast mussels that were classed as developing were clustered together, and were distributed away from parasites and granulomas meaning that these were of low prevalence and intensity in developing mussels (Figure 5d).

Key for NMDS Plots

(a.) Site: **1** Bannow Bay, **2** Flaxfort Strand.

(b.) Sex: **0** Indeterminate, **1** Male, **2** Female.

(c.) Month: **3** March, **4** April, **5** May, **6** June, **7** July, **8** August, **9** September, **10** October, **11** November, **12** December, **13** January, **14** February, **15** March, **16** April, **17** May, **18** June.

(d.) Stage of gonadal maturity: **0** no gonads, **1** early developing, **2** late developing, **3** ripe, **4** spawning/partially spent, **5** spent/resorbing, **6** resorbed.

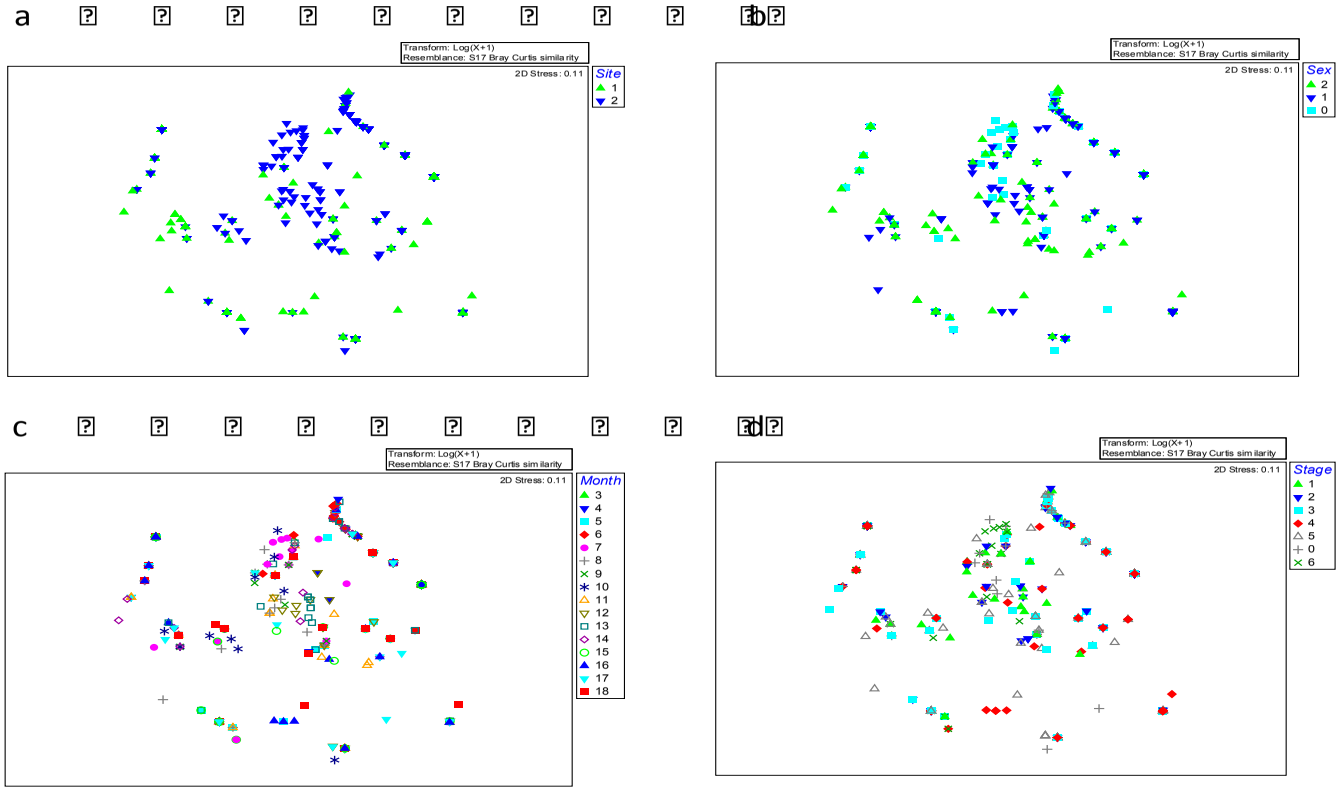


Figure 5: Non-metric multidimensional scaling plots showing (a.) site, (b.) sex (c.) month and (d.) stage of gonadal maturity.

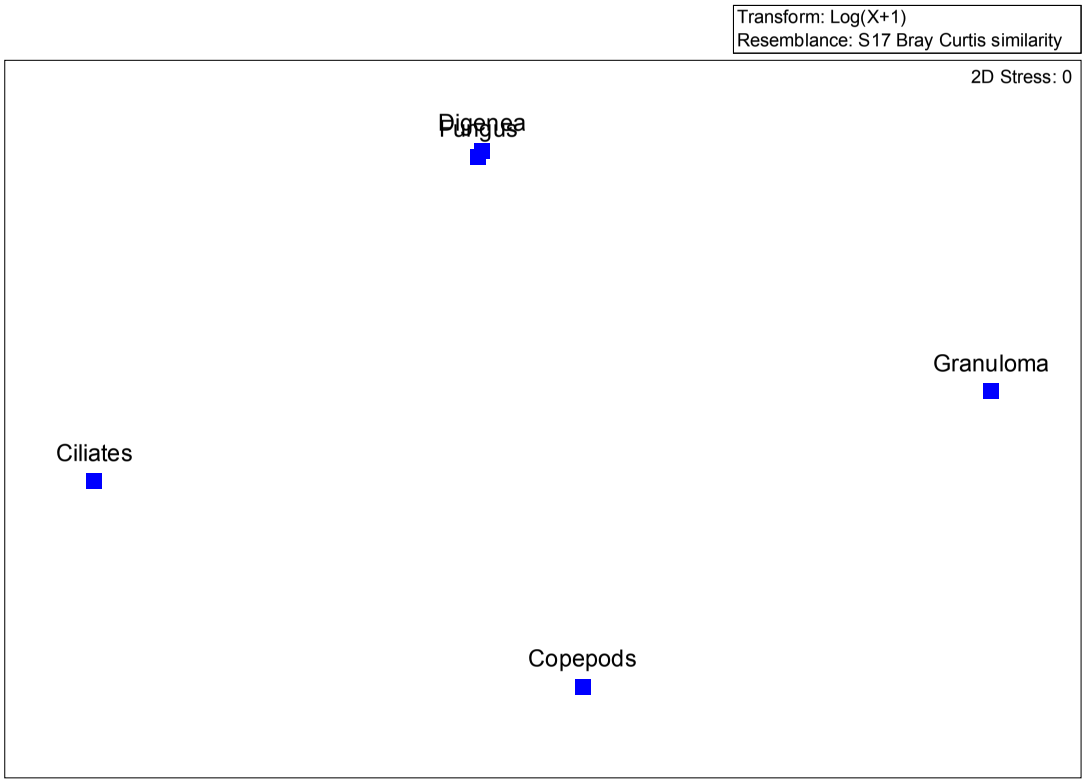


Figure 6: Variable non-metric multidimensional scaling ordination.

6.4 DISCUSSION

Parasitism in mussels does not inevitably mean that they are in poor health. A range of parasites, and to a lesser extent morphological changes, were recorded in the present study, however but no abnormal mortalities were observed and a concurrent study of mussel reproduction confirmed this too is on-going normally at both sites (Chapter 3).

In comparison to other histological studies of mussel pathology (e.g. Cremonte *et al*., 2005; Bignell *et* al., 2008; 2011), lower prevalence of trematodes, ciliates, *Mytilicola intestinalis*, and *Nematopsis* sp. oocysts were observed. Although the prevalence of trematode metacercariae in Flaxfort Strand was significantly greater than at Bannow Bay, mussels from Flaxfort Strand underwent two spawning events compared to one at Bannow Bay and were significantly greater in terms of length, width, height, whole weight, shell weight, and tissue weight than those from Bannow Bay (Chapter 3). Parasitism is an antagonistic relationship and the added stress it causes can impact on mussels' ability to adapt to alterations in ambient conditions such as temperature (Calvo–Ugarteburu and McQuaid, 1998) and under stressful conditions mussel growth can be reduced (Wilson *et al*., 2011) however the levels of parasitism recorded in the present study do not appear to be having such impacts. The prevalence or intensity may not be great enough i.e. the threshold required for disease to develop may not have been reached, or the interactions and combinations of parasites are such that high pathogenicity is not expressed.

Previous studies of mussel parasites in Europe and South America have utilised both whole body squash preparations and histology to assess parasite diversity and burden (Table 5). Although some similarities can be seen in the parasite fauna identified in each study there were some parasites unique to each site identified. For example *Polydora ciliata* were identified from the Wadden Sea only, whereas *Trichodinia*-like ciliates and *Nematopsis* sp. oocysts were only recorded in Ireland. Similarly, coccidians were confined to Southampton/Devon and ciliophora to the Tamar Estuary (Bignell *et al*., 2008; 2011). Variation over a smaller spatial scale was apparent also, with a greater range of parasites in mussels collected from Flaxfort Strand than Bannow Bay and NMDS analysis together with ANOSIM displayed that these sites were significantly different overall.

Table 5: The range of parasites and morphological changes recorded in *Mytilus edulis* from previous studies of its histopathology.

Site	n	Parasite Species	Morph. Changes	Method	Ref.
Southern Norway	max. 240	Digenean metacercariae	Haemocyte aggregations	Histopathology and Histochemistry	Aarab *et al*., 2011
		Steinhausia mytilovum	Digestive tubule atrophy		
		Marteilia sp.	Lipofuscin		
			Neutral lipids		
List Tidal Basin, Wadden Sea	234	*Himasthla elongata*	n/a	Whole Squash Preparation	Thieltges *et al*., 2006
		Himasthla continua			
		Himasthla interrupta *Psilostomum brevicolle* *Renicola roscovita*			
		Gymnophallus gibberosus *Paravortex cardii*			
		Mytilicola intestinalis Unknown Copepod			
		Polydora ciliata			
Tamar Estuary and Trebarwith Strand	150	*Rickettsia*-like organisms	Apoptosis	Histology	Bignell *et al*., 2011
		Ancistrum mytili or other ciliates	Atresia		
		Gregarine	Inflammation		
		Metaceracariae	Granulocytoma		
		Marteilia sp.	Brown cell inflammation		
		M. intestinalis	Pearl formations		
		Ciliophora -like organism			
Southampton and Devon, UK	1200	*Rickettsia*-like organisms	Atrophy	Histology	Bignell *et al*., 2008
		Bucephalids	Melanised bodies		
		Protozoan	Eosinophilic bodies		
		Haplosporidia	Degeneration		
		M. intestinalis	Melanised aggregates		
		S. mytilovum	Haemocytic neoplasia		
		Marteilia sp.	Gonadal apoptosis		
		Metaceracariae	Atresia		
		Copepod	Granulocytoma		
		Coccidian	Inflammation		
		A. mytili or other	Haemocyte aggregation		

		ciliates Gregarine	Pearls		
Archachon Bay, France	338	*H. elongata* *H. continua* *H. interrupta* *P. brevicolle* *R. roscovita* *P. cardii* *M. intestinalis*		Whole Squash Preparation	de Montaudouin *et al*., 2000
San Jose Gulf, Argentina	30	Basophilic bacteria *Rickettsia*-like bacteria Ciliates		Histology	Cremonte *et al*., 2005

Parasite species present in a given area may be governed by several factors such as tidal height, pollution, upstream host distribution, and the dilution effect - "non-hosts interfering with the transmission of free-living parasite stages, so less abundant in downstream hosts" (Hechinger and Lafferty, 2005; Johnson *et al*., 2008; Thieltges *et al*., 2012). The dilution effect may be particularly apparent in Bannow Bay, where there was a lower diversity and abundance of parasites but a greater number of bivalve taxa observed than in Flaxfort Strand including additional species such as *Ruditapes* sp. and *Crassostrea gigas* (see Chapter 6 – section 2). Variation over a large scale may be linked to environment; while over a local scale biological factors may be of greater importance, with distance decay evident in parasite fauna similarity (Wilson *et al*., 2011). Additionally the parasite fauna are undoubtedly linked to presence of final hosts of some of the species, or other migrations of organisms in the ecosystem (Marcogliese and Cone, 1997). Inter-host differences may be apparent also due to the size, age, immune status of the host and the interactions of various parasite species contained within it (Thieltges *et al*., 2012).

Due to their high intensity, bucephalid trematode sporocysts can cause extensive tissue damage and castrate the host, which has negative ramifications for cultivation and commercial exploitation (da Silva *et al*., 2002). However overall they were identified in one mussel only from Flaxfort Strand only; their prevalence was lower than metacercariae but the intensity observed was very high in this one individual, which is a common phenomenon, reported in bivalve – parasite systems (Fermer 2010; Morgan *et*

al., 2012). Sporocysts have been absent too in other studies of mussel parasites using squash preparations (de Montaudouin *et al*., 2000) and in histological sections sporocysts were absent in other studies from the Tamar Estuary (Bignell *et al*., 2011) and Patagonia (Cremonte *et al*., 2005).

Variation among trematode species in prevalence and intensity may be related to upstream and downstream hosts (Combes, 1991). Both sites are protected under the EU Birds Directive due to the abundance of birds, and the final host of each the metacercariae species identified is a waterbird. In the present study three species of metacercariae were identified. *Gymnophallus choledochus* was the most common trematode at both sites, and its first and final hosts; *Cerastoderma edule* and waterbirds, are abundant at Bannow Bay and Flaxfort Strand. However, a higher density of *C*. *edule* in Flaxfort Strand (personal observation) may be reflected in the significantly higher prevalence and intensity of the parasite at this site. Similarly this too could be said of *Himasthla interrupta* and *H*. *elongata*. *Himasthla interrupta* were more common, and this may be due to the abundance of the upstream host, *Hydrobia ulvae* whose populations at both sites could reach high levels as they colonised surfaced moribund cockles. They were often found in close proximity to mussels, sharing the substratum that the mussels had settled on (personal observation). The first intermediate host of *H*. *elongata*, *Littorina littorea* was less abundant and conspicuous at both sites. Studies of *Cerastoderma edule* have shown that the density of the upstream host, in this case again *Hydrobia ulvae* and *Littorina littorea*, was of key importance for downstream host parasite intensity (Wilson *et al*., 2011).

Interestingly *Trichodinia*-like ciliates were found in histological sections of mussels from Flaxfort Strand only. *Trichodinia*-like ciliates are known to become endoparasitic entering the digestive glands of hosts when intertidal conditions become difficult (Mladineo *et al*., 2012). There is controversy too concerning the pathogenicity of copepods such as *Mytilicola intestinalis* and the ciliate *Ancistrum mytili*, which were found at both sites at low levels in the study (Fuentes *et al*., 1995; Villalba *et al*., 1997; da Silva *et al*., 2002; Rayyan *et al*., 2006). Often they are considered commensals or symbionts of mussels, but a reduction in condition may be associated with them under unfavorable environmental conditions and high intensities (Rayyan *et al*., 2006)., which were not observed in the present study.

An unidentified parasite, believed to be a fungus was also observed, being more common at Flaxfort Strand. Marine fungi can be toxic for bivalves especially in the larval stages. Previous studies of mussels and cockles at both sites have shown that despite spawning occurring at both sites in these species, there is a lack of younger, smaller animals at Flaxfort Strand (Morgan *et al*., 2012; Chapter 3; Chapter 4). The prevalence of this fungus-like parasite was positively correlated with water temperature at Flaxfort Strand. As the Irish Sea is increasing in temperature at six times the global average (Frost *et al*., 2012) and European Seas were classed as "rapid warming" in a recent study (Belkin, 2009) this fungus may represent an emerging disease, whose pathogenicity is facilitated by climate change. Marine fungi may also interact with shellfish phycotoxins increasing their toxicity and studies have shown that the frequency and range of harmful phytoplankton blooms are increasing with rising sea surface temperature (Belgrano *et al*., 1999; Matallah-Boutiba *et al*., 2012).

Fungi and digeneans appeared to co-occur together as indicated by NMDS ordination. Infection of mussels by opportunistic secondary bacteria and fungi may occur through puncture marks or damaged siphons that trematodes make while colonising the host. No immune response was evident to the fungal growths, despite the sometimes-large size of the fungus.

Several pathologies and morphological changes were observed. These responses may be indicative of normal immune response in the face of parasitism or idiopathic (e.g. granuloma) or a reaction to some kind of environmental stressor in the case of lipofuscin granules. Lipofuscin granules in the mantle can be related to the presence of metals, as can lipofuscin granules in the organs especially if they are in the kidney (Bignell *et al*., 2011). They were present at both sites, but at low levels in comparison to other studies e.g Bignell *et al*. (2008; 2011). Recent studies have concentrated on the levels of various metals, organometals and PAHS present in the tissues of finfish and mussels around Ireland. Tyrrell *et al*. (2005) analysed the levels of mercury, cadmium, chromium, copper, lead and zinc in fish from five major fishing ports – Howth, Rossaveal, Killybegs, Dunmore East (59.1 km from Bannow Bay) and Castletownbere (112 km from Flaxfort Strand). Nickel, lead, cadmium, and chromium were not detected in tissues of finfish at either site. While mercury, copper and zinc were low and under the EU limits as set under European Regulation 466/2001/EC. This partly agrees with a study performed on mussels throughout Ireland (and the United

Kingdom), which compared the scope for growth and contaminant levels (Widdows *et al*., 2002). In agreement with Tyrrell *et al*. (2005), concentrations of metals in tissues were all below the "no observed effects threshold" in Ireland (Widdows *et al*., 2002). Therefore the presence of lipofuscin may indicate low levels of metals that are being processed by the mussels but not impacting upon them deleteriously.

Differences between months could be related to inherent seasonal cycles in mussel physiology and reproduction, which are influenced by temperature and genotype (Bignell *et al*., 2008). Other studies of mussels have shown some seasonality in lipofuscin accumulation, granulomas, and copepods (Bignell *et al*., 2008; Aarab *et al*., 2011). However no such seasonal trend was observed in bucephalid trematodes (da Silva *et al*., 2002). Variation between months could be dependent on parasite species present, mussel physiology, and reproductive state in combination with local environmental conditions.

Stage of gonadal maturity was also highlighted as a source of variation between mussels, with animals at different stages of maturity exhibiting different parasites. This could interact with site difference; mussels from Flaxfort Strand have two spawning periods, whereas only one has been recorded in Bannow Bay (see Chapter 4). Mounting an immune response is an energetically costly process, as is undergoing reproduction (Taskinen and Saarinen, 1999). In the case of the freshwater clam *Anondonta piscinalis* and its copepod gill parasite *Paraergasilus rylovi,* female clams that are reproducing have a greater parasite burden than those that do not (Taskinen and Saarinen, 1999). It has been suggested that copepod survival could be enhanced in reproducing clams as immunocompetence may be reduced by reproduction (Taskinen and Saarinen, 1999) this too could be the case for mussels from Flaxfort Strand. Alternatively increased need for nutrients to fuel two spawning periods may mean that mussels from Flaxfort Strand have a higher filtration rate, which could offer a route for colonization by trematode metacercariae (Nikolaev *et al*., 2006). But it should be noted that despite significantly greater prevalence and intensity of metacercariae at Flaxfort Strand, two spawning events occurred there so the energy demands of trematodes on mussels might not have been that great.

There was a much higher prevalence of infection in mussels from Flaxfort Strand; this indicates that there was a much higher biomass of parasites present. Easier transmission of parasites may be facilitated at this site by a greater number of susceptible hosts,

which have been impacted upon by environmental stressors, as Flaxfort Strand is of poor water quality compared to Bannow Bay. However parasite intensity is comparable between the sites and it is quite low especially in comparison to other studies. The majority of mussels from Bannow Bay were not parasitised and of those that did have parasites the majority had single infections with low intensities only. The majority of parasitised individuals at Flaxfort Strand again had one infection of parasites only and as in Bannow Bay with a low intensity. After a threshold level of parasites is reached in a host, then the host-parasite relationship is altered such that mortalities occur (O'Grady *et al*., 2013). In the case of mussels from both sites, due to the prevalence of single infections, and the low intensities observed, it does not appear that this threshold has been reached. Additionally for disease and subsequent mortalities to occur there need to be the correct combination of host, parasites and external environmental parameters. This combination does not appear to have been achieved at either site leading to parasitism having a low or reduced effect on the hosts (Hedrick, 1998).

In conclusion, although several parasites and pathologies were identified in the course of this study, they were in comparatively low prevalence and intensity of infection and unlike the common cockle at the study sites, no "abnormaly" high mortalities were observed during the study. Indeed several studies describe *Trichodinia*-like ciliates as a commensal and the pathogenicity of *Mytilicola intestinalis* is debated. Additionally no notifiable pathogens that may lead to a reduction in mussel productivity were recorded. Although morphological changes were recorded at low levels that may be indicative of low levels of environmental pollution, and other studies of contaminants in the Irish Sea have shown that levels of many pollutants are low. Therefore it could be argued are parasitized mussels really unhealthy? Trematode sporocysts were found in mussels from Flaxfort Strand only, plus metacercariae were significantly more prevalent and intense (but still at a low level) at this site than Bannow Bay. However mussels at Flaxfort Strand were significantly greater in all size parameters measured and had two spawning events – which means that they are still successfully accruing nutrients, are fecund and growth rates were not being impacted upon. This is in comparison to other bivalves, which may experience parasitic castration or loss of gonadal tissue (Valderrama *et al*., 2004). Therefore it could be concluded that being healthy and being parasitised are not mutually exclusive, at least in the case of the mussels in this study.

Maybe as a threshold level was not reached, or the interactions between mussel, environment and parasites did not create the conditions for it to occur.

SECTION 2: Parasite diversity across all hosts.

6.5 ABSTRACT

Synecology – the study of whole animal communities-especially of soft sediments are lacking. Parasites although associated with all free-living organisms, are often an under reported or ignored source of biodiversity in intertidal and estuarine areas and little is known concerning the range of and distribution of parasites in many non-commercial species and few studies have concentrated on entire ecosystems (e.g. de Montaudouin *et al*., 2000). Trematodes may dominate the intertidal area in terms of parasite type, and have consequences for the species that they inhabit and all the species that the host encounters which in turn may impact on shaping community structure. Full inventories of parasites in particular ecosystems are lacking. The parasite fauna of several bivalve species was therefore investigated at two sites in southern Ireland. Fieldwork was performed at monthly intervals for 16 months, and all bivalves encountered were collected and examined using histology. In total 3,073 bivalves were studied comprising *Cerastoderma edule*, *Mytilus edulis*, *Macoma balthica*, *Angulus tenuis*, *Scrobicularia plana*, *Mya arenaria* and a *Ruditapes* specimen. The greatest range of parasites in terms of diversity and abundance was identified in *C. edule* and then *M. edulis*. In total one pathological condition - disseminated neoplasia plus 22 parasites (macro and micro) were recorded. *Nematopsis* sp. oocysts had the greatest number of potential host species (5); while others were restricted to one host only e.g. *Paravortex cardii* in *Cerastoderma edule* or *Ancistrum mytili* in *Mytilus edulis*. Variation was apparent between the species in terms of parasitic fauna, plus their prevalence and intensity. No parasites were recorded in *M. arenaria* – however this may be as a consequence of small sample size but trematode metacercariae were recorded in the one *Ruditapes* collected. In short, the parasite fauna of several bivalves in one ecosytem were described in Ireland for the first time, with variation between species apparent.

6.6 INTRODUCTION

Wetlands such as estuaries are often characterised by high biodiversity (Junk *et al*., 2012), however much of this diversity may be overlooked as it is in the form of parasites (Marcogliese and Cone, 1997; Thieltges *et al*., 2009). This is despite the majority of taxa being afflicted by parasites, and all food webs containing parasites

(Marcogliese and Cone, 1997; Poulin, 1999; Thieltges *et al*., 2009). Studies of the biology and parasite fauna of many non commercial species is lacking, as are studies comparing the parasite fauna across a number of taxa in one ecosystem (de Montaudouin *et al*., 2000; Thieltges *et al*., 2006). Furthermore Bordes and Morand (2009) have pointed out that studying one host parasite system is thought to underestimate the complexity, impacts and interactions of multiple parasite species.

For every free-living organism, there is, using conservative estimates, at least one parasite (Poulin, 1999; Bordes and Morand, 2009). Parasitism has been described as a type of symbiotic relationship, however the parasite derives energy and exerts harm on the host (Thieltges *et al*., 2012). Parasites are being increasingly recognised as sources of biodiversity within intertidal and estuarine communities, as is their influence on hosts, which then may shape communities. Broadly, parasites can be subdivided into macro and micro parasites. Macroparasites in general are multicellular, with complex life cycles and long generation times e.g. trematodes, cestodes, and polychaetes (Thieltges *et al*., 2012). In contrast microparasites are unicellular, with simple lifecycles and direct transmission to conspecifics e.g. fungi, protozoa, bacteria and viruses (Thieltges *et al*., 2012). The presence of certain parasites may too be suggestive of greater biodiversity. Parasites, in particular those with complex life cycles, such as digenean trematodes are indicative of the presence of other hosts e.g. birds, in that area (Marcogliese and Cone, 1997).

As previously mentioned the parasite fauna of many bivalves has not been investigated, for example non -commercial species like *Angulus tenuis*, the thin tellin. As a result in many studies biodiversity and food web connectance are currently conservative and underestimated due to lack of presence of the parasite fauna. Therefore in the present study the parasite fauna across a number of bivalve species in the intertidal area was investigated.

These little studied bivalves may too act as reservoirs of infection until transmission to the preferred host. There are multiple definitions of reservoirs of infection (see Small and Pagenkopp, 2011), but in this experiment they were considered as any invertebrate that is capable of carrying, maintaining and transmitting a parasite (Small and Pagenkopp, 2011). There may be many reservoirs of parasites in natural systems (Small and Pagenkopp, 2011).

The ubiquity of parasitism and parasites cannot be denied and they then affect their host and the host's subsequent interactions (Thieltges *et al*., 2012). Similarly despite much attention being given to commercial bivalves in estuaries e.g. *Cerastoderma edule*, there can be a wide range of other bivalves that contribute to ecosystem services e.g. *Macoma balthica*. Despite each of these species of bivalve being widely distributed and contributing to the ecosystem as a whole; as food sources and links between trophic levels, not much attention has been given to their health in Ireland. The overall aim of the present study was to gain a greater understanding of soft sediment communities, firstly by investigating the various bivalve's parasite fauna as there is little record of parasitological studies of these non-commercial species and for comparative purposes, as they inhabit the same areas as the main species under study in this thesis *Cerastoderma edule* and *Mytilus edulis*. As parasites influence hosts, who then are part of the wider ecosystem their prevalence and intensity is of great interest (Poulin, 1999; Thieltges *et al*., 2009).

6.7 RESULTS

3,073 bivalves were included in the study. In total one pathological condition - disseminated neoplasia, and 24 parasites were identified. The parasite fauna was dominated by digenea, as 10 species of trematode were identified, some digenea were restricted in host species (e.g. *Meiogymnophallus minutus*) while others e.g. *Himasthla interrupta* had a number of host species. Four species of ciliate, in addition to four types of bacterial growths, two types of copepod, a cestode, a turbellarian plus a gregarine and a fungus were recorded too. No parasites were identified in the soft shell clam *Mya arenaria*.

6.7.1 *Disseminated neoplasia*

The pathological condition - disseminated neoplasia was identified in the cockle *Cerastoderma edule* only. Two distinct cell types were seen: one light with discernible cell contents, and the other with darker chromatin in an off centre nucleus.

The mean prevalence of neoplasia in surfaced cockles from Bannow Bay was 21.8 ± 16.25% and in buried cockles it was 6.4 ± 6.12%. Neoplasia was significantly more prevalent in surfaced rather than buried cockles (Mann-Whitney Test $P = 0.007$). The prevalence of neoplasia ranged from 6.7% in November 2010 to 46.7% in August 2010

in surfaced cockles and from 3.3% (September 2010, March 2011 and June 2011) to 17.2% in June 2010 in buried cockles.

There was a higher mean prevalence of neoplasia in surfaced cockles (27.1 ± 20.18%) than buried cockles (4.2 ± 5.69%) at Flaxfort Strand and this proved significant (Mann-Whitney Test P = 0.003). Prevalence of neoplasia ranged from 6.7% in February 2011 to 53.3% in June 2011 among surfaced cockles, whereas the highest prevalence among buried cockles was in May 2010 (20%) and it was at a low in July 2010, August 2010, and March 2011 (3.3%). The latter stages of neoplasia were most common in surfaced cockles, and during the summer months. Neoplasia was identified in cockles from 8.1 mm – 38.1 mm in height, and at both sites it was most prevalent in cockles of intermediate length or with three growth rings.

6.7.2 *Trematode Sporocysts*

It was not possible to screen trematodes by squashing the whole animal tissue as individuals were being screened for a range of parameters. Therefore prevalence of infection in bivalves was determined from screening tissue sections only, but all bivalves were treated the same. The chosen method was histology – and as only sections of tissues from the animal are screened an underestimation of intensity may occur and the species cannot be determined conclusively unlike when using squash preparations when these pitfalls can be avoided.

Sporocysts in cockles were identified as *Labratrema minimus*, as this species was recorded previously in Courtmacsherry Bay and on the east coast. There was a higher prevalence of sporocysts in buried cockles from both sites (BB s 1.2 ± 2.17 %: b 1.6 ± 2.14 %, FF s 1.0 ± 1.92 %: b 1.1 ± 2.0 %). The prevalence ranged from 3.3 % (October 2010) to 6.7 % (April 2011) in surfaced cockles from Bannow Bay, and from 3.3 % (July, October 2011, January and March 2011) to 6.7 % in May 2011 in buried cockles at the same site. Among surfaced and buried cockles in Flaxfort Strand prevalence of sporocysts ranged from 3.3 % in late summer 2010 and 2011 to 6.7 % in June 2010 (surfaced) and 6.7 % in October 2010.

Despite a higher prevalence of sporocysts in buried cockles at both sites, the intensity was greater in surfaced cockles, and comparing sites, higher in Bannow Bay than Flaxfort Strand (BB s 71.0 ± 126.57: b 50.2 ± 86.38 sporocysts per section, FF s 48.0 ± 93.07: b 30.0 ± 66.86 sporocysts per section). The highest intensity of sporocysts in surfaced cockles in Bannow Bay was July 2010 (365 ± 0 sporocysts per section) and in

January 2011 in buried cockles (291 ± 0 sporocysts per section). The lowest intensity of sporocysts in surfaced cockles from Bannow Bay was in October 2010 (140 ± 0 sporocysts per section) and in buried cockles was 25 ± 0 in July 2010.

Bucephalid trematodes were identified in one mussel from Flaxfort Strand in December 2010 only (overall mean prevalence 0.22 %; mean intensity 46.6 ± 180.48 sporocysts per section).

Trematode sporocysts were identified in *Angulus tenuis* in June 2010 from Flaxfort Strand but due to the high intensity the numbers could not be quantified. *Scrobicularia plana* from Flaxfort Strand only had sporocysts. The sporocysts identified in *S. plana* may have been *Meiogymnophallus minutus* and were at high intensity (99 sporocysts per section).

6.7.3 *Trematode Metacercariae*

Several species of metacercariae were recognised during the course of the study, they were present at all sites, but in varying prevalence and intensities per host species.

Meiogymnophallus minutus metacercariae were identified in cockles. *M. minutus* were the most common species in cockles with high prevalence at both sites (BB s 59.1 ± 12.72 %: b 47.2 ± 21.28 %, FF s 68.8 ± 27.87 %: 64.6 ± 20.16 %). The prevalence of *M. minutus* ranged from 40 % in surfaced cockles in Bannow Bay (April and May 2010) to 83.8 % (November). *M. minutus* prevalence ranged from 13.3 % in May 2011 to 80 % (March 2010, June 2011) in buried cockles from Bannow Bay. Prevalence of *M. minutus* at Flaxfort Strand ranged from 37.9 % in April 2011 in surfaced cockles, to 90 % in October 2010, compared with 46.7 % in May 2010 and June 2011 to 86.7 % in September 2010. The greatest intensity of *M. minutus* metacercariae was in surfaced cockles from Bannow Bay (BB s 33.2 ± 15.69: b 23.3 ± 12.46 metacercariae per section, FF s 27.7 ± 12.80: 28.1 ± 11.31 metacercariae per section). The intensity of *M. minutus* ranged from 2.8 ± 2.94 metacercariae per section from buried cockles in May 2011 from Bannow Bay to 70.0 ± 74.67 metacercariae per section in February 2011 in surfaced cockles. In comparison cockles from Flaxfort Strand had an intensity of trematodes ranging from 12.4 ± 13.39 metacercariae per section in buried cockles to 50.1 ± 47.86 metacercariae per section in surfaced cockles. A trematode, which resembled *Meiogymnophallus minutus*, was identified in the sole *Ruditapes* specimen screened also.

Metacercariae of *Himasthla interrupta* were identified in cockles and mussels. They were identified in the gonads and digestive system of cockles. There was a higher prevalence and intensity of *Himasthla interrupta* in cockles from Bannow Bay than from those collected in Flaxfort Strand (BB s 12.3 ± 12.15 %: b 11.1 ± 13.81 %, FF s 3.6 ± 4.68 %: 2.8 ± 2.75 %). The prevalence of *H. interrupta* ranged from 3.3 % in surfaced (June 2010) and buried cockles (June, July 2010 and May 2011) to 33.3 % in surfaced (May 2011) and 40 % in buried cockles (February and March 2011) in Bannow Bay. At Flaxfort Strand, the prevalence of trematodes ranged from 3.3 % in surfaced and buried cockles (Spring/Summer) to 13.8 % in surfaced cockles collected in September 2010 and 8 % in January 2011.

Himasthla interrupta had a significantly higher mean prevalence (BB 5.6 ± 4.75 %: FF 26.1 ± 17.34 %) and a significantly greater mean intensity (BB 1.2 ± 1.12 metacercariae per section: FF 2.6 ± 1.26 metacercariae per section) in mussels from Flaxfort Strand than Bannow Bay (prevalence Mann Whitney U test, P = 0.001, intensity Independent Samples t-test, P = 0.003). It ranged in prevalence from 3.3 % (March, May, August 2010 and February, April 2011) to 14.3 % in November 2010 in Bannow Bay. There was a much higher maximum prevalence in Flaxfort Strand at 63.3 % in December 2010 and a low of 3.3 % in October 2010 and May 2011. The highest intensity of *H. interrupta* was in June 2010 from Flaxfort Strand (5.8 ± 5.11 metacercariae per section).

Himasthla elongata was recorded in mussels only and colonised mainly the foot and connective tissues. Its mean prevalence (Mann Whitney U test, P = 0.008) and intensity (Mann Whitney U test, P = 0.003) were significantly higher in Flaxfort Strand than Bannow Bay (mean prevalence BB 0.9 ± 2.42 %: FF 9.5 ± 11.17 %; mean intensity BB 0.1 ± 0.35 metacercariae per section: FF 2.26 ± 2.38 metacercariae per section). The highest prevalence overall was in June 2010 from Flaxfort Strand (37.9 %) and the highest intensity was in October 2010 (7.5 ± 7.77 metacercariae per section).

A possible *Himasthla* spp. was identified in *Macoma balthica*, *Angulus tenuis* and *Scrobicularia plana*. There was a higher, but not significant difference in the mean prevalence of trematode metacercariae (possible *Himasthla* spp. and unknown species combined prevalence) in *Macoma balthica* from Bannow Bay (7.6 ± 6.60 %) compared to Flaxfort Strand (3.5 ± 5.81 %). The highest prevalence of metacercariae occurred in January 2011 (20 %) in Bannow Bay and April 2010 (11.1 %) in Flaxfort Strand, lows were experienced in March 2011 (5 %) in Bannow Bay and July and October 2010 (3.3

%) in Flaxfort Strand. The intensity of trematode metacercariae was greater at Bannow Bay too (BB 1.6 ± 1.95 metacercariae: FF 0.5 ± 0.65 metacercariae). The greatest intensity of metacercariae was recorded in April 2010 in Bannow Bay, and July 2010 in Flaxfort Strand. The mean prevalence of metacercariae was greater in *A. tenuis* from Flaxfort Strand (7.8 ± 17.15 %) rather than Bannow Bay (2.9 ± 4.50 %) but not significantly so. Prevalence of metacercariae was highest in April 2010 at both sites in *A. tenuis* (BB 9.1 %: FF 50 %). Trematode intensity was greater in Flaxfort Strand (0.6 ± 0.78 metacercariae) than Bannow Bay (0.5 ± 0.83 metacercariae). Possible *Himasthla* spp. trematode metacercariae were identified in *S. plana* (Prevalence 0.4 ± 0.54 %; intensity 0.4 ± 0.54 metacercariae) and the highest prevalence was in February 2011 (100 %).

Metacercariae were identified as *Gymnophallus choledochus* due to their presence in the gonads, the formation of a pearlised structure, and their previous identification in bivalves in Ballycotton, Ireland (Fermer *et al*., 2011). *Gymnophallus choledochus* was the least common trematode recorded in cockles, it was found at a low prevalence, and was recorded only in buried animals from Bannow Bay (0.6 ± 1.33 %) in November 2010, March and May 2011, but surfaced in Flaxfort Strand (0.2 ± 0.85 %) in September 2010. Reflecting its low prevalence, very low intensities of this parasite were also recorded (BB 0.06 ± 0.25 metacercariae per section: FF 0.1 ± 0.34 metacercariae per section).

However it was the most prevalent metacercariae in mussels at both sites and significantly more so at Flaxfort Strand (Mann Whitney U test, P = 0.001) (BB 12.6 ± 8.90 %: FF 36.2 ± 15.59 %). They had a significantly higher mean intensity in Flaxfort Strand (2.2 ± 1.08 metacercariae per section) than in Bannow Bay (1.2 ± 0.94 metacercariae per section) (Independent Samples t – test, P = 0.014). In mussels they ranged in prevalence from 3.3 % (February 2011) in Bannow Bay to 36.7 % (April 2010), and from 13.8 % (March 2011) to 66.7 % (February 2011) in Flaxfort Strand.

6.7.4 *Nematopsis sp. oocysts*

Oocysts of the apicomplexan gregarine *Nematopsis* sp. were identified. *Nematopsis* sp. was extremely prevalent in cockles at each of the sites (BB s 83.2 ± 16.11 %: 75.0 ± 27.96 %, FF s 85.8 ± 15.75 %: 89.7 ± 13.68 %). Its prevalence was greater in surfaced cockles from Bannow Bay, and its intensity was significantly greater at this site. In

contrast the prevalence and intensity of *Nematopsis* sp. was higher but not significantly so in buried cockles from Flaxfort Strand.

The prevalence of *Nematopsis* sp. ranged from 23.3 % (May 2010) to 100 % (February 2011) in buried cockles from Bannow Bay and from 60 % (April 2010) to 100 % (September 2010 and January 2011) in surfaced cockles at the same site. The highest intensity recorded was in surfaced cockles in Febuary 2011 from Bannow Bay (104.4 ± 102.19 per section) and the lowest was in buried cockles in Bannow Bay in June 2010 (2.9 ± 3.52 per section).

In mussels there was a higher prevalence of *Nematopsis* sp. from Flaxfort Strand (2.0 ± 2.82 %) than at Bannow Bay (0.4 ± 1.16%) but they did not differ significantly (Mann Whitney U test, P = 0.174). It was present in March only of both years at Bannow Bay, but in all seasons in Flaxfort Strand. *Nematopsis* sp. also had a higher mean intensity at Flaxfort Strand (0.5 ± 0.71 oocysts per section) than Bannow Bay (0.2 ± 0.56 oocysts per section) (Mann Whitney U test, P = 0.905)

There was a higher but not significant difference in the mean prevalence and intensity of *Nematopsis* sp. between *Macoma balthica* collected from both sites (Prevalence BB 1.1 ± 3.51 %: FF 0.2 ± 0.88 %; intensity BB 0.2 ± 0.63 oocysts: FF 0.1 ± 0.26 oocysts).

Nematopsis sp. were more prevalent in *A. tenuis* from Flaxfort Strand, but there was a greater intensity at Bannow Bay (Prevalence BB 2.2 ± 3.56 %: FF 2.6 ± 5.44 %, intensity BB 0.8 ± 1.60 oocysts: FF 0.2 ± 0.42 oocysts).

The greatest prevalence and intensity of *Nematopsis* sp. in all species under study was in *S. plana*, there was a higher prevalence in Bannow Bay (56.7 ±± 43.46 %) than Flaxfort Strand (32.9 ± 43.47 %). However there was a higher intensity in Flaxfort Strand (18.8 ± 47.30 oocysts) than Bannow Bay (5.0 ± 5.14 oocysts).

6.7.5 *Ciliates*

Trichodinia-like and *Rynchodida*-like ciliates were identified, as were *Ancistrum mytili* and ciliates of unknown species.

The prevalence of ciliates of unknown species was 9.7 ± 7.09 % in mussels from Bannow Bay and 7.6 ± 8.13% in Flaxfort Strand.

Trichodinia-like ciliates were recorded in cockles from both sites, but with a higher prevalence in Flaxfort Strand (BB s 4.5 ± 9.11: b 4.9 ± 5.59 %, FF s 10.6 ± 9.36: b 12.7

± 13.9 %). They were also catalogued in mussels from Flaxfort Strand only but at much lower prevalence (0.2 ± 0.92 %) and intensity (0.1 ± 0.26 ciliates).

Rynchodidia-like ciliates were recorded in cockles and there was a higher prevalence in Flaxfort Strand (s 7.0 ± 4.57: b 7.1 ± 5.40 %) than at Bannow Bay (s 3.3 ± 4.14 %: b 2.7 ± 2.58 %.

The gill ciliate *Ancistrum mytili* was identified in mussels from both sites, it was more prevalent with a higher intensity at Flaxfort Strand but not significantly so (prevalence Independent Samples t-test, P = 0.642, intensity Mann Whitney U test P = 0.061) (prevalence BB 2.1 ± 4.46 %: FF 2.7 ± 2.63 %, intensity BB 0.3 ± 0.45 ciliates per section: FF 1.0 ± 1.00 ciliates per section).

Macoma balthica surveyed from Flaxfort Strand had three species of trematode (*Trichodinia*-like, *Rynchodida*-like and unknown species) whereas those from Bannow Bay were parasitised by *Trichodinia*-like cilates and unknown species only. There was no significant difference in the mean prevalence of ciliates (all combined) between Bannow Bay (0.2 ± 0.42 %) and Flaxfort Strand (1.0 ± 1.41 %) in *M. balthica*. The highest prevalence of ciliates was recorded in June 2010 (13.3 %) in Bannow Bay and December 2010 (100 %) in Flaxfort Strand.

Angulus tenuis from both sites though were parasitised by *Rynchodida*-like cilates (and unknown species) only with equal prevalences (BB 1.8 ± 3.54 %: FF 1.8 ± 2.11 %). The highest prevalence and intensity of ciliates recorded in *A. tenuis* at Bannow Bay was in April 2010 (prevalence 36.4%, intensity 9.0 ± 13.39 ciliates).

6.7.6 *Cestodes*

Cestodes of unknown species were recorded in cockles and mussels, in Bannow Bay only for cockles and Flaxfort Strand only for mussels. The prevalence of cestodes in cockles was 0.2 ± 0.88 %, they were identified in June 2010 only and had a mean intensity of 0.1 ± 0.26 cestodes per section.

In mussels, cestodes were found exclusively at Flaxfort Strand, and in January and February 2011 only. The prevalence was 0.4 ± 1.16 % and they had an intensity of 0.1 ± 0.35 cestodes per section.

6.7.7 *Mytilicola intestinalis*

Mytilicola intestinalis and another less common unidentified copepod were identified in mussels at both sites. There was a higher prevalence of the copepod *M. intestinalis* in Flaxfort Strand than Bannow Bay (prevalence BB 5.0 ± 6.44 %: FF 8.9 ± 7.63 %) which

proved to be non-significant (Mann Whitney test, P = 0.052), the intensity was comparable between sites and again non-significant (Independent Samples t-test, P = 0.905) (BB 0.8 ± 0.59 copepods per section: FF 0.8 ± 0.46 copepods per section). The highest prevalence of *M. intestinalis* was apparent in May 2010 in Bannow Bay, whereas highs were recorded in late autumn and winter in Flaxfort Strand and absent at this site in the summer months.

6.7.8 *Copepod unknown species*

An unknown copepod species was more prevalent and intense at Bannow Bay (prevalence 0.5 ± 1.29 %, intensity 0.13 ± 0.35 copepods per section) than Flaxfort Strand (prevalence 0.2 ± 0.89 %, intensity 0.06 ± 0.25 copepods per section) but not significantly so (Mann-Whitney U test, P = 0.744). It was recorded twice in Bannow Bay in July 2010 and November 2010, but only in October 2010 in Flaxfort Strand.

6.7.9 *Paravortex cardii*

The turbellarian *Paravortex cardii* was identified in surfaced and buried cockles from Flaxfort Strand and surfaced cockles only from Bannow Bay, with a higher prevalence and intensity in buried cockles (prevalence BB s 0.2 ± 0.88 %: b 0, FF s 0.2 ± 0.88: 0.7 ± 1.37 %). The intensity was greatest in cockles from Flaxfort Strand (BB s 0.1 ± 0.26 turbellarians per section: b 0, FF s 0.1 ± 0.26: b 0.3 ± 0.59 turbellarians per section).

6.7.10 *Unidentified Fungus*

An unidentified fungus was recorded in cockles, mussels, Baltic tellins and thin tellins. It stained blue/purple and could form large oval branching structures. No immune response was observed around it. At Bannow Bay and Flaxfort Strand, the fungus was more prevalent in surfaced cockles at both sites (BB s 2.1 ± 3.09: 0.4 ± 1.72 %, FF s 6.5 ± 5.91:b 2. ± 3.39 %). In mussels the fungus had a marginally higher non-significant mean monthly prevalence in Flaxfort Strand (16.8 ± 13.49 %) than Bannow Bay (16.6 ± 11.31 %) and was at its most prevalent in summer months (Independent Samples t-test, P = 0.965). The unidentified fungus was recorded in samples of *Macoma balthica* from Flaxfort Strand (4.9 ± 8.9 %) and in *Angulus tenuis* from both sites (BB 0.8 ± 1.94 %: FF 0.3 ± 1.04 %).

6.7.11 *Rickettsia-like organisms*

Rickettsia-like organisms (RLOs) associated with gill filaments were seen at both sites in mussels, but at a higher prevalence in Bannow Bay (2.3 ± 3.14 %) than Flaxfort Strand (1.4 ± 2.17 %) (Mann Whitney U test, P = 0.595). The intensity of RLOs was

greater also in Bannow Bay (BB 1.1 ± 1.91 RLO per section: FF 0.4 ± 0.63 RLO per section).

6.7.12 *Eosinophilic rod-like bacteria – unidentified bacteria one*

Eosinophilic bacteria were recorded in cockles and Baltic tellins. The prevalence was greatest in surfaced cockles from Flaxfort Strand (BB s 2.4 ± 3.55: b 2.4 ± 3.45 %, FF 3.1 ± 3.33: 1.9 ± 2.36 %). Rod shaped eosinophilic bacteria were identified adjacent the digestive area in *Macoma balthica* from both sites but in higher prevalences in Bannow Bay (0.83 ± 2.62 %) than Flaxfort Strand (0.2 ± 0.88 %).

6.7.13 *Grey rod-like bacteria – unidentified bacteria two*

Grey rod-like bacteria were recorded in surfaced cockles from Flaxfort Strand only (0.7 ± 1.40 %).

6.7.14 *Gill Cysts enclosing bacteria*

Gill Cysts enclosing bacteria were recorded in surfaced cockles from Bannow Bay only with a low prevalence (0.2 ± 0.88 %).

6.8 DISCUSSION

In total the parasites of seven bivalve species were investigated in the current study, with one pathological condition and 24 different parasites were identified. It was clear that *Cerastoderma edule* and *Mytilus edulis* play host to a wider range of parasites than the other bivalves, and that some parasites may have the ability to use different bivalves as a host e.g. *Nematopsis* sp. The present study showed the diversity of parasites present in bivalves in the intertidal region. However no parasites were recorded in *Mya arenaria* at either site, this may be related to the depth in the sediment at which they are normally found which is deeper than the position that they were excavated from in the present study (de Montaudouin *et al*., 2000).

Disseminated neoplasia (DN) was identified in the present study and it was confined to cockles, in other studies it has been recorded in *Mytilus edulis* and *Macoma balthica* too (Rasmussen, 1986; Sokolowski *et al*., 2004). In this present study two types of neoplastic cells were identified – which is in agreement with previous studies of DN in cockles (Twomey 1987; Carballal *et al*., 2001; Morgan *et al*., 2012). Both cell types could be seen in the one cockle. It has been theorised that these cell types could indicate two cell lineages or a progression of the disease (Carballal *et al*., 2001).

Neoplasia was significantly more prevalent in surfaced cockles from both Bannow Bay and Flaxfort Strand. This has been identified previously in Flaxfort Strand in 2009 (Morgan *et al*., 2012) and further afield in Arcachon Bay, France (Le Grand *et al*., 2010). Neoplasia is usually a lethal disease (House *et al*., 1998; Collins and Mulcahy, 2003; Ciocan and Sunila, 2005), and surfacing in cockles has been described as both a "prelude to death" and an "indicator of poor health" (Blanchet *et al*., 2003). Therefore surfaced cockles could be in the final stages of the disease and no longer have the ability to right their position in the sediment. A concurrent study assessed the population structure and gametogenesis of cockles at sites. The condition index, which may be viewed as a proxy or good indicator for health was calculated (Calvo-Ugarteburu and McQuaid, 1998; Orban *et al*., 2002) and it was significantly less in surfaced cockles at both sites. This again stresses the ill health of surfaced cockles in comparison to those that remain buried. The method by which neoplasia is lethal is as yet unknown, but it could be linked to secondary infection due to the inability of neoplastic cells to mount an immune response (Ciocan and Sunila, 2005), or starvation again linked to loss of phagocytic ability in the haemocytes.

Neoplasia is a progressive disease and variations in severity can be seen (Ciocan and Sunila, 2005). Neoplasia was staged according to a scale from Collins and Mulcahy, (2003). Stage 0 was no neoplastic cells in the sections ranging to stage 4, which was the most severe final stage of the disease. The later stages of the disease were more common in surfaced cockles and in the summer and late autumn at both sites. This seasonality in intensity of the disease could be linked to the temperature. Stage 1 neoplasia was most common in buried cockles at both sites, neoplasia is thought to be in populations year round, and perhaps this is how it persists at the early stages not causing mortality (Barber, 2004) also previous studies have indicated that moderately affected animals can either go into remission or die (Twomey and Mulcahy, 1988; Ciocan and Sunila, 2005). The progression of the disease to the fatal stage in summer months could be associated with temperature stress.

The parasite fauna was dominated by species of trematodes and 10 species were identified. Some (e.g. *Himasthla elongata*) were restricted in host, while others (e.g. *Himasthla interrupta*) had a wider host distribution. A large number of trematode species has been identified in the Dutch Wadden Sea (Thieltges *et al*., 2006) when the macroparasitic fauna of bivalves was compared. Host phylogeny is believed to play a

part in determining the parasites species of a host, and hosts with a common evolutionary history may have similar parasite assemblages (Vickery and Poulin, 1998). *Cerastoderma edule* and *Mytilus edulis* both harboured a range of parasites while the soft shell clam *Mya arenaria* had none. This may be reflective of firstly the positioning on or in the sediment, as *M. arenaria* buries much deeper than cockles or mussels. In a similar vein, the possibility of a parasite with a complex life cycle being transmitted to the next host would be greater in cockles or mussels as they are more readily available to predators. Although the other bivalves examined (*Macoma balthica*, *Angulus tenuis* and *Scrobicularia plana*) were parasitized, they had a lesser range of species; this may be due to their small size or lower densities than cockles and mussels, which may impede transmission. They also may not be the preferred host, and could be the "diluting" rather than "amplifying" host as transmission to the final host may be impeded (Rohr *et al*., 2011). However the low prevalence and intensities observed may again indicate that these were parasitised opportunistically rather than preferentially; as parasites infect all susceptible hosts that they encounter (Brooks and Hoberg, 2007). Plus the interactions between the various parasite species in a host can affect the parasite species present (Vickery and Poulin, 1998). As cockles have previously been described as a series of microhabitats (de Montaudouin *et al*., 2009) perhaps cockles and mussels are large enough to have enough space for partitioning between parasites to reduce interactions, especially negative ones.

Some parasites e.g. ciliates, *Nematopsis* sp., and fungus infection had a wide number of host species, while some others, in particular trematodes, were restricted to one host. Additionally no host immune response was noted, even when a high intensity of *Nematopsis* sp. was recorded; in contrast, host bivalves were seen to be encapsulating trematodes. Perhaps the restriction of trematodes in host species is governed by their adaptations to one particular host environment and immune response and switching successfully between hosts is not feasible (Brant and Loker, 2005). Specialising on one host species is advantageous as it allows for the creation of enzymes, attachment systems and the ability to mitigate the host's immune system (Combes, 1997).

In the majority of cases the species of parasite could be discerned, however in the case of cestodes, copepods and ciliates, unidentified species were present. Considering the potential deleterious implications of mortalities and loss of condition especially in commercially exploited species, perhaps more attention should be paid to examining

parasite species and their impact on the host. Widening the focus on parasites beyond histology and squash preparations to the use of molecular tools would provide more classification on the species present (as in Chapter 8)

The present study emphasised the diversity of parasite species in the intertidal region. Parasites are present in all food webs, but may not be recognised as such (Marcogliese and Cone, 1997). The range and abundance of parasites encountered in the present study was substantial: parasites however are often a little recognised source of diversity, and may have a important influence on their hosts and in their interaction with each other. This may come about through requiring energy from the host, behavioural changes, increased mortality, lowered fecundity, reduced growth, modification of the sex ratio, and alterations to competition in general (Marcogliese and Cone, 1997; Vickery and Poulin, 1998). Therefore it can be argued that no study of an ecosystem is complete without considering the parasite fauna too.

Variation between the parasite fauna of each site was observed too. This dissimilarity may be attributed to the presence of other suitable hosts in an area, particularly the final host (Marcogliese and Cone, 1997: Hechinger and Laffery 2005). This can offer clues to the other species inhabiting the area, however ephemeral (Marcogliese and Cone, 1997). As both sites are important feeding areas for a range of bird species (and are designated under the Birds Directive) as such the diversity and abundance of trematodes is to be expected. The variation observed in parasite fauna between sites may be due to environmental factors – as the conditions at one site are unlikely to be replicated at another, especially if they are separated spatially by over 100 km (Thieltges *et al*., 2009; Wilson *et al*., 2011). The presence of other hosts, substratum and degree of exposure influence the parasite fauna of an area too, and all these factors differ between Flaxfort Strand and Bannow Bay (Wilson *et al*., 2011).

The identification of trematodes, ciliates and fungi in the species under study may indicate that they can act as reservoirs of infection, maintaining these taxa in the environment if their preferred host species is of low density or abundance. They may be of particular importance to parasites, especially as population declines in bivalves, such as the cockle *Cerastoderma edule* have been reported due to milder winters attributed to climate change (Philippart *et al*., 2003). Reservoir species could retain certain parasites in an ecosystem, if the preferred or usual host is experiencing declines.

In conclusion, one pathological condition and a wide range of parasites were recorded in the course of the study. The ubiquity, high prevalence, and high intensity of certain parasites emphasized their importance and how much they contribute to overall diversity of the study areas. Additionally it showed that these parasites may have interactions with each other as several parasites were found in one host, and may provide additional information as to how they may influence interactions between hosts of the same or different species as parasites may alter host growth, fecundity, sex ratio and mortality.

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CHAPTER 7: EVEN THEIR PARASITES HAVE PARASITES:ATTEMPTS TO DETERMINE GENETIC DIVERSITY IN A HOST – PARASITE – HYPERPARASITE SYSTEM.

7.1 ABSTRACT

The population structure of *Cerastoderma edule*, its parasite *Meiogymnophallus minutus*, and in turn its hyperparasite the microsporidian *Unikaryon legeri* were investigated across Europe and North Africa. Low levels of genetic variation in cockles across Europe have been reported. However the question was posed, is this true also for one of its parasites and in turn, hyperparasite, as the migrations of the parasite's final host, the oystercatcher, may mean the parasite and its hyperparasite may be genetically more diverse than the cockle. Samples of cockles were collected from Ireland, Wales, France and Morocco. Trematodes were visualised in the cockle and then DNA was extracted using Qiagen (Cork and Morocco) and Chelex (all other sites) extractions. A single nucleotide polymorphism was apparent in cockles between Cork and Morocco. Despite repeated attempts with oligonucleotides targeting different regions (18Scom, ITS, CO1) of the trematode, amplification was unsuccessful. Generic microsporidian primers were used to amplify *Unikaryon legeri* but only reverse sequences were rendered. This study shows the difficulty in amplifing these taxa, molecular studies are lacking for them, and the taxonomy of microsporidia is based on morphology only. The possible variation in DNA quality was highlighted by using the different extraction methods as Qiagen extracted DNA only amplified products, PCR inhibitors may have been present in Chelex extracted DNA resulting in PCR failure.

7.2 INTRODUCTION

Cerastoderma edule, the common cockle, is a widely distributed, abundant infaunal bivalve mollusc. It is ecologically and commercially important throughout its range from Mauritania in West Africa to northern Norway, is commercially fished and is a key member of the soft sediment ecosystem (Martínez *et al*., 2009; Krakau *et al*., 2012; Martínez *et al*., 2012; Morgan *et al*., 2012). Therefore understanding the gene flow between cockles is integral for future management plans and to define populations. Previous studies have investigated the population genetics of this species in Europe using allozymes, the cytochrome oxidase 1 (CO1) region and microsatelites (Beaumont and Pether, 1996; Martínez *et al*., 2009; Coscia *et al*., 2012 Krakau *et al* 2012).

Beaumont and Pether (1996) utilised allozyme studies to determine gene flow and therefore variation among allozyme loci between populations of cockles in the United Kingdom. Cockles from eight sites in the south of the United Kingdom were included in the study, these consisted of western sites: Peele Harbour (Isle of Man), Treath Melynog (Anglesey), Dee Estuary (Greenfield), Dwyryd Estuary (Portmadog) and Burry Inlet (Llanelli) and the eastern sites: Toff Sands (The Wash), Southend Foreshore (Thames) and East Barrow Sand (Thames). It was found that there were high levels of polymorphic loci and heterozygosity. There were also consistent deficiencies of heterozygotes against Hardy-Weinberg Model assumptions. Significant geographic allele variations were seen for all populations except at the *Gpi* locus, but there was little differentiation and therefore gene flow among cockle populations (Beaumont and Pether, 1996). However, cockles from Burry Inlet in South Wales were genetically different from other populations (Beaumont and Pether, 1996). Subsequent studies in Europe have indicated that there is a substantial amount of larval dispersal among cockles and this was indicated using allozyme studies (Andre *et al*., 1999) and mitochondrial CO1 analysis (Krakau *et al*., 2007). Following CO1 analysis, one dominant haplotype was identified in *C*. *edule* from populations that extended from Norway to Morocco (excluding the Wadden Sea) (Krakau *et al*., 2007). The recognition of one dominant haplotype in the study performed by Krakau *et al*. (2007) further verifies the hypothesis that larval drift/distribution of *C*. *edule* is extensive. A more recent study performed by Coscia *et al*. (2012) is again in agreement with high dispersal capacity of cockle larvae. The study combining the usage of microsatellites and biophysical modelling to compare genetic diversity and population structure between Ireland and Wales showed that there was connectivity between Irish and Welsh sites as allele frequencies were similar (Coscia *et al*., 2012).

Martínez *et al*. (2009) developed 12 microsatelite markers to differentiate genetic diversity throughout the extensive range of the cockle. Three to 17 alleles were observed, significant heterozygote deficiency was apparent at five loci which also had null alleles, whereas the other seven adhered to the Hardy-Weinberg equation (Martínez *et al*., 2009). A further study by Martínez *et al*. (2012) using 11 of these markers, comparing cockles from Scotland, the Netherlands, France,

Spain and Portugal, again found heterozygote deficiency that they attribute to the presence of null alleles. Observed heterozygosity across all samples was lower than expected and allelic richness decreased at higher latitudes. Cockles collected from Portugal and Spain (except from one site, Ferrol) were found to be homogenous in nature, whereas those from France, the Netherlands and Scotland were different from each other and from the southern populations due to distance and the presence of barriers (Martínez *et al*. 2012). They did not find evidence for a nothern refugia which is in contrast with Krakau *et al*. (2012) who amplified a different region, the mitochondrial cytochrome oxidase 1 gene. Evidence for a northern glacial refugia is provided by Krakau *et al*. (2012) in their study comparing the CO1 gene in cockles from Murmansk, Russia to as far south as Merja Zerga, Morocco. They believe that there is a northwestern and a southwestern population present, that were separated in antiquity due to high genetic diversity in the northern sites compared to low diversity and differentiation in the southern sites (Krakau *et al*., 2012). Additionally the authors of that study believe that the southern population of cockles is younger than the northern population and a distinct Arctic population may also be present (Krakau *et al*., 2012).

Throughout its range *Cerastoderma edule* is known to be parasitised by 16 species of digenean trematodes, in addition to turbellarians and copepods (de Montaudouin *et al*., 2009). Eight species of digenean trematode are known to utilise *C*. *edule* as a first or second intermediate host in Ireland and the digenean trematode *Meiogymnophallus minutus* is one of the most common parasites of *Cerastoderma edule* found throughout its range and in especially high intensities and prevalences on Irish shores (Fermer, 2009). *Meiogymnophallus minutus* is a member of the Family Gymnophallidae, subfamily Gymnophallinae of the Phylum Platyhelminthes which may include up to 24,000 species (Bowers and James, 1967; Moszczynska *et al*., 2009). *Meiogymnophallus minutus* metacercariae remain unencysted in the host (Fermer *et al*., 2009), however its tegument undergoes changes during transition from cercariae to metacercariae and it becomes covered by alternating rows of transverse small spines (Bowers and James, 1967; Russell-Pinto and Bowers, 1998). *Meiogymnophallus minutus* produces metacercariae that are smaller than other digeneans found in Europe

and are 0.12 – 0.300 mm in length. Their small size has been linked to ease of emergence from the first host or alternatively; it allows for the production of a large number of smaller cercariae (Fermer, 2009). They are found in the hinge region of cockles (Goater, 1993). The metacercaria possesses a Y shaped excretory vesicle, which enlarges and becomes full of excretory granules as the metacercariae develop (Bowers and James, 1967).

Unlike the usual digenean life cycle in which a gastropod mollusc is the first intermediate host, and the subsequent host is a bivalve, *Meiogymnophallus minutus* also utilises a bivalve, the peppery furrow shell *Scrobicularia plana* as a first host, and the oystercatcher *Haematopus ostralegus,* the common scoter *Melanitta nigra* and eider ducks *Somateria mollisma* as definitive hosts (Russell-Pinto, 1990; Goater, 1993). Due to the migratory nature of the bird final host, greater diversity may be apparent in *M. minutus* if they are transported with the final host as they undergo migrations.

In several studies *Meiogymnophallus minutus* has been infected by the microsporidian hyperparasite *Unikaryon legeri* (Bowers and James, 1967; Azevedo and Canning, 1987; Goater, 1993; Russell-Pinto and Bowers, 1998; Fermer *et al*., 2009). Trematodes that are classified as hyperparasitised are thought to be easily distinguishable from non-parasitised (Goater, 1993) and hyperparasitism appears to exclusively occur in metacercariae, not in the sporocysts or the cercariae (Fermer, 2009). Hyperparasitism would be beneficial for the original host, the cockle, as it eventually kills the trematode thus curtailing completion of the life cycle and it is thought to spread from infected to uninfected metacercariae (Fermer *et al*., 2009).

Microsporidia have undergone several taxonomic revisions since their first description in the literature in 1857 as a yeast-like fungus, before inclusion as early branching eukaryotes due to their widespread origins (Corradi and Keeling, 2009; Smith, 2009). However once again they are classified as a fungus, possibly with zygomycete ancestry (Smith 2009; Ryan and Kohler, 2010) due to the chitin rich spore that they produce and the formation of an intranuclear spindle during mitosis (Smith, 2009). They are obligate, intracellular parasites, which display vertical, horizontal, and mixed transmission strategies (Hirt *et al*.,

1999; Van der Peer *et al*., 2000; Garcia, 2002; Corradi and Keeling, 2009; Ryan and Kohler, 2010; Wilkinson *et al*., 2011). 140 to 160 genera of microsporidia have been described (Garcia, 2002; Corradi and Keeling, 2009; Smith, 2009) and they are believed to infect all major animal groups, being found in insects, oligochaetes, fish and mammals but individual species have narrow host ranges (Garcia, 2002; Smith, 2009). Thus far their classification has been based upon morphology, cellular structure and host specificity with minor amounts of molecular classification (Corradi and Keeling, 2009; Smith, 2009; Wilkinson *et al*., 2011).

The mode of infection employed by microsporidia is highly specialised and uses dedicated unique apparatus (Van de Peer *et al*., 2000) (Figure 1). The life cycle has two stages, one a proliferative merogonic stage followed by sporogony (Garcia, 2002). Microsporidia form spores, which are the only free living stage and have no metabolic activity when not enclosed in a host cell (Garcia, 2002; Corradi and Keeling, 2009). Spores are enclosed by chitin and protein (Van de Peer *et al*., 2000) and contain a polar tubule (Garcia, 2002). The polar tubule is a coiled projectile apparatus, which everts when the spore reaches a potential host (Van de Peer, 2000; Garcia, 2002; Corradi and Keeling, 2009). The spore's cytoplasm is then injected into the host's cytoplasm through the tubule, the parasite then continues to grow and multiply creating more spores (Van de Peer, 2000). Eversion of the polar tubule is achieved by increase of pressure caused by water entering the spore through aquaporins (Corradi and Keeling, 2009). As some microsporidia e.g. *Dictyocoela duebenum* the parasite of *Gammarus duebeni* are vertically transmitted only, feminisation and subsequent sex ratio distortion (SRD) of hosts has been reported (Smith, 2009; Ryan and Kohler, 2010; Wilkinson *et al*., 2011). Embryos become infected and then develop as females, their survival is not impaired, but growth rates are reduced (Smith, 2009).

Eleven species of the *Unikaryon* genus have been described with common features; disporoblastic sporogeny, unpaired nuclei at all stages and development in contact with host cytoplasm are all considered traits of the genus. All members of this genus are known from Platyhelminthes (including *Unikaryon legeri*) (Sene *et al*., 1997). Several other microsporidia from the genera

Unikaryon and *Nosema* are reported as hyperparasites, for example *Nosema dollfusi* the hyperparasite of *Bucephalus cuculus* which itself parasitises *Crassostrea virginica* (Levron *et al*., 2004).

Thus far using various markers (e.g. microsatellites, allozymes) the connectivity of cockle populations has been shown except when separated by a great distance or by barriers such as currents (Krakau *et al*., 2012). In general all aforementioned studies make reference to the ability of the cockle to disperse. In the present study the population structure of *Cerastoderma edule* was analysed using the 18Scom region and cockles from both northern and southern latitudes were included. Due to their ecological and commercial importance, determining populations of *C*. *edule* is integral for their future management. It was hypothesised that some variation may be evident in the population genetics of the parasites and hyperparasites that utilise this species, which may help define populations of *Cerastoderma edule*. There is a paucity of literature concerning the trematode *Meiogymnophallus minutus* in terms of genetics, so this too offered an opportunity to examine this further. Similarly this can be said also of *Unikaryon legeri*, whose classification is based entirely on non-molecular traits and has undergone several revisions. Similarly "molecular genetic studies provide exceptional insight into relationships, migration and evolution of natural populations" (Seeb *et al*., 2011).

7.3 MATERIALS AND METHODS:

7.3.1 *Sample Collection*

Cockles were collected from a number of locations in Europe and Morocco (Table 1). Those from Cork were kept alive in aquaria at ambient temperature until required. The tip of the foot of each cockle was removed, and then the remainder of the body was cut into three pieces, which were then placed between glass plates and compressed. *Meiogymnophallus minutus*, which was infected with *Unikaryon legeri*, was then identified in the tissues using microscopy and a guide (de Montaudouin *et al*., 2009). They were extracted from the tissues using a pipette and forceps and placed in 70 % alcohol to preserve. Cockles from Morocco and France were provided in 70 % alcohol using the same technique outlined above. Samples of cockles from other Irish locations and Wales were frozen until required then defrosted slowly and the foot and adductor muscle was removed for DNA extraction.

Table 1: Location of collection points.

Location	Latitude
IRELAND	
1. Dundalk, Co. Louth	N 53°59'52.60" W 6°24'25.45"
2. Sandymount, Co. Dublin	N 53°19'50.00 W 6° 12'56.67"
3. Bannow Bay, Co. Wexford	N 52°13'07.9" W 006°46'37.8"
4. Flaxfort Strand, Co. Cork	N 51°38' 47.4" W 008°41'45.0"
5. Glenbeigh, Co. Kerry	N 52° 03'25.87" W 009° 58'03.60"
WALES	
6. Red Wharf Strand, Anglesey	N 53° 18'21.32" W 4°10'28.10"
7. Swansea Bay, Swansea	N 51° 34'22.31" W3°59'22.41"
FRANCE	
8. Somme	N 50°14'46.35" E1°34'55.57"
MOROCCO	
9. Merja Zerga, Morocco	N34°50'39.98" W6°16'10.06"

7.3.2 *DNA Extraction*

DNA from Flaxfort Strand and Morocco were extracted using a Qiagen DNeasy Blood and Tissue Kit using the "Purification of total DNA from animal tissues (Spin Column Protocol)" technique. All other sites were extracted using a Chelex 10 % reaction (Walsh *et al*., 1991; Lynch *et al*., 2008). Extracted DNA was kept frozen until required. Tissue sections were cut and then pulverized using a scalpel and forceps before the extraction process began. DNA concentrations were checked using a NanoDrop Spectrometer ND 1000. 1.5 µl of DNA was used each time, and AE buffer, which had been used to resuspend DNA, was used as a blank sample.

Figure 1: Map of study locations with numbering corresponding to table 1.

7.3.3 *Polymerase Chain Reaction*

All reactions were performed on a Hybaid Thermocycler and double distilled water was used as a negative control. Stage one of each reaction denatured the DNA, annealing of the DNA occurred in stage two and stage three was the extension step. Any resultant PCR products amplified were visualised on a 2% agarose gel containing 15 µl of ethidium bromide (10 mg/ml) stock, after electrophoresis. Resulting bands were measured against Qiagen GelPilot 100bp ladder. MWG Eurofins performed direct sequencing on pooled (same individual, n = 3) unpurified PCR products; generated sequences, forward and reverse, were then compared to the NCBI nucleotide database with BLASTn to confirm species.

7.3.4 *Cerastoderma edule*

The 18Scom region of *Cerastoderma edule* was amplified using 18ScomF1 and 18ScomR1 primers (Table 2) (Zhang *et al*., 2005) with modified thermocycling conditions (Lynch, unpublished data).

Table 2: Primers used to amplify *Cerastoderma edule*.

Name	**Primer**	**Region**	**Reference**
18ScomF1	5'-GCTTGTCTCAAAGATTAAGCCATGC-3'	18Scom	Zhang *et al*., 2005
18ScomR1	5'-CACCTACGGAAACCTTGTTACGAC-3'	18Scom	

7.3.5 *Meiogymnophallus minutus*

Meiogymnophallus minutus was targeted for amplification using several methodologies. The cytochrome oxidase subunit 1 (CO1) was amplified using JB3 (Bowles *et al*., 1993 as read in Leung *et al*., 2009) and trem.cox1.rrnl (Králová-Hromadová *et al*., 2001). Three different PCR mastermixes were used and two different thermocycling conditions (Appendices 1, 2 and 3). The 18s region of the trematode was amplified using 18Scom F1 and R1 primers (Zhang *et al*., 2005) with modified thermocycling conditions (Lynch unpublished data) (Appendix 4). Medlin *et al*. (1988)'s 16S-like rRNA primers were utilised to amplify Eukaryotic 16S-like r RNA-coding regions of the trematode (Appendix 5). The ITS region of the trematode was targeted using Warberg *et al*. (2005)'s primers (Appendix 6).

Table 3: Primers used to amplify *Meiogymnophallus minutus*

Name	Primer	Region	Reference
JB3	5'-TTTTTTGGGCATCCTGAGGTTTAT-3'	CO1	Leung *et al*., 2009
trem.cox1.rrnl	5'-AATCATGATGCAAAAGGTA-3'		
18ScomF1	5'-GCTTGTCTCAAAGATTAAGCCATGC-3'	18Scom	Zhang *et al*., 2005
18ScomR1	5'-CACCTACGGAAACCTTGTTACGAC-3'		
EUBA F1	5'-AACCTGGTTGATCCTGCCAG-3'	SSUrRNA	Medlin *et al*., 1988
EUBB R1	5'-TGATCCTTCTGCAGGTTCACCTAC-3'		
BDA-2	5'-GCGCTGAGAAGACGACCAAA-3'	ITS	Warberg *et al*., 2005
ITS2JH	5'-GCTGCGCTCTTCATCGACAC-3'		

7.3.6 *Unikaryon legeri*

Unikaryon legeri was amplified using V1 (forward) and 1492 (reverse) primers for *Nosema* spp. (Wilkinson *et al*., 2011) (Table 4). The reaction was performed twice using the same master mix and conditions (Appendix 7).

Table 4: Primers used to amplify *Unikaryon legeri*

Name	Primer	Reference
v1 (forward)	5'-CACCAGGTTGATTCTGCCTGAC-3'	Wilkinson *et al*., 2011
149 (reverse)	5'-GGTTACCTTGTTACGACTT-'3	

7.4 RESULTS

Table 5: DNA quantification:

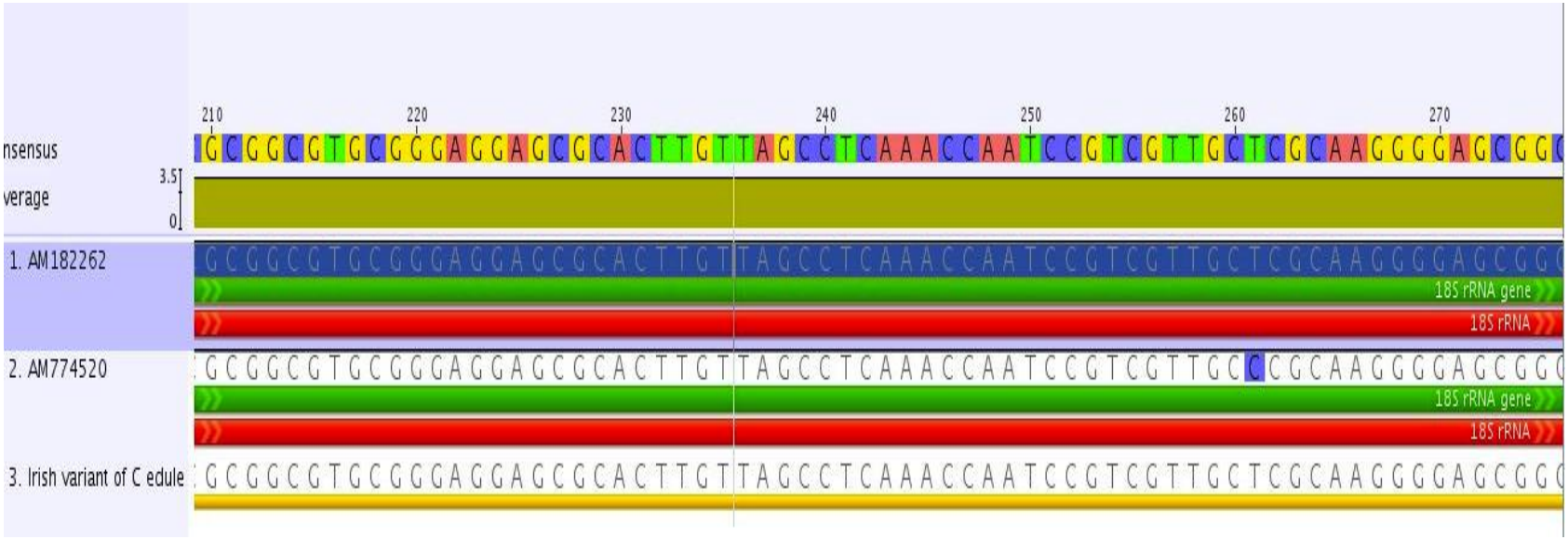
Sample	DNA concentration	Sample	DNA concentration
	ng/ul		ng/ul
Flaxfort Strand		**Merja Zerga**	
1	62.46	**1**	72.04
2	71.92	**2**	299.5
3	117.87	**3**	39.44
4	203.4	**4**	393.59
5	166.66	**5**	229.73
6	70.77	**6**	138.63
7	238.02	**7**	134.04
8	46.87	**8**	92.45
9	304.14	**9**	300.67
10	93.48	**10**	30.59
11	165.33	**11**	56.07
12	82.3	**12**	102.28
13	225.51	**13**	84.78
14	167.2	**14**	177.09
15	192.36	**15**	34.76

7.4.1 *Cerastoderma edule* amplification and sequencing.

Products corresponding to cockle DNA were amplified at 900bp using the 18s primers and were direct sequenced by MWG Eurofins. Both forward and reverse DNA sequences were obtained for three individuals. BLASTn analysis was carried out and a single nucleotide polymorphism (SNP) was observed in the sequenced samples. Cytosine in cockles from Cork was replaced by thymine in those from Morocco at 261 bp. Comparison against the Genbank type for cockle 18s region (AM774520, Taylor *et al*., 2007) showed that this SNP differed from AM774520 (Taylor *et al*., 2007) but had been recorded before (Genbank AM18226, Pradillon *et al*., 2007). Successful amplification was only achieved using Qiagen extracted DNA (Table 5) not Chelex extracted DNA.

Even their parasites have parasites.

Table 6: *Cerastoderma edule* sequences comparing Cork versus Genbank types.



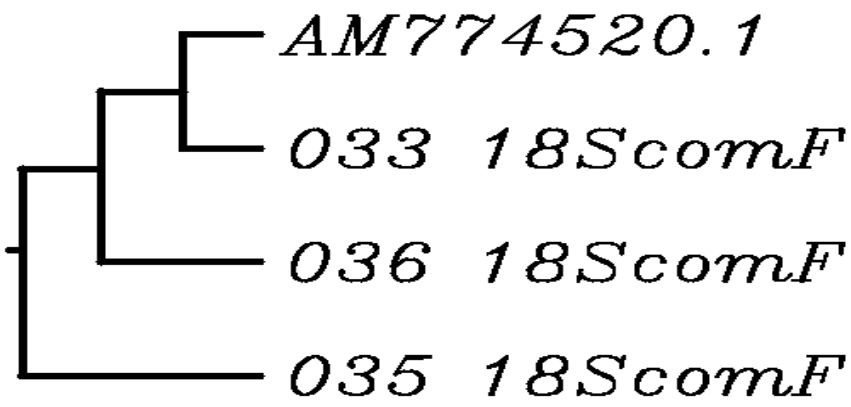


Figure 2: Phylogenetic tree comparing the cockles used in the present study (33, 35 & 36) to the Genbank type AM774520.1.

7.4.2 *Meiogymnophallus minutus* amplification and sequencing

It was not possible to amplify *Meiogymnophallus minutus* using the above methodologies.

7.4.3 *Unikaryon legeri* amplification and sequencing

Two attempts were made to sequence *Unikaryon legeri*-using primers described in Wilkinson *et al*. (2011). In the first PCR product only reverse sequences could be recovered from the PCR product, in the second PCR product mixed sequences were found.

7.5 DISCUSSION

This research highlighted the lack of molecular data concerning both trematodes and microsporidia. Analysis of *Cerastoderma edule* was slightly more successful and single nucleotide polymorphisms were observed between Cork and Morocco.

A single nucleotide polymorphism was observed between the sites. These are polymorphisms at individual nucleotide sites and such occurences in protein

expression may be related to disease development including tuberculosis, various cancers and neoplasia in humans (Beebee and Rowe, 2005; Caws *et al*., 2008; Einstein *et al*., 2009) however further research is required in bivalves. The SNP observed may be related more so to local adaptation.

Sequencing of cockle DNA was only possible from Qiagen extracted individuals not Chelex extracted. The efficiency of the widely used Chelex extraction has been argued (Casquet *et al*., 2012). However it is best used with fresh samples and despite yielding DNA it could be of low quality and contain PCR inhibitors that cause suboptimal DNA preservation or PCR failure (Casquet *et al*., 2012). Whereas Qiagen extractions although more expensive, produce higher quality DNA but in low amounts (Casquet *et al*., 2012). Perhaps the time span for which cockle tissues were stored, although they remained frozen, may have impacted on DNA quality.

Successful amplification of target DNA relies upon several factors including host tissue, optimum primer design, and sufficient Taq polymerase and nucleotides (dNTPs). Both the trematode and the microsporidian were visualised in the samples prior to DNA extraction. Post extraction, the DNA was quantified and high levels were uniformly observed. However, this high level of DNA may have corresponded to the cockle host (which was repeatedly amplified). The method of recovering trematode metacercariae from host tissue may have impeded further experimentation too. Cockle tissues were compressed between two glass plates, and the area of tissue with trematodes removed and preserved. Multiple trematodes could be present in any one section. Inducing cockle hosts to shed trematodes using light and heat may be more prudent, removing a potential source of interference (cockle tissue).

Perhaps the levels of trematode DNA were too low to amplify, however in a previous study using CO1 primers to barcode *Diplostomum* and *Tylodelphys* specimens from birds, fish and amphibians, Moszczynska *et al*. (2009) found that the amount or quality of trematode DNA did not impact upon primer performance.

In the present study several different primers were utilised to amplify the CO1, 18s and ITS regions of the *Meiogymnophallus minutus* genome. The CO1 primers that were utilised had been used successfully in studies of trematodes in New Zealand (Leung *et al*., 2009) and also for *Fasciola* spp. (Králová-Hromadová *et al*., 2008). However, previous studies have concluded that creation of CO1 primers for the CO1 region of Platyhelminthes may be problematic, as there is a high level of sequence divergence (Moszczynska *et al*., 2009) despite other studies showing that the CO1 region can be used across a broad number of taxa (Kress *et al*., 2005).

The usefulness of the ITS1 region in trematode phylogenetic studies has been examined, using trematodes isolated from arthropods and *Hydrobia ulvae* (Schulenberg *et al*., 1999; Warberg *et al*., 2005). It was found that due to 5'end variability, secondary structure conservation and scrutiny of derived characters, that digenean taxonomic hierarchy may be deduced from the ITS1 region (Schulenberg *et al*., 1999). It may in addition be used to decipher species complexes (Bartoli *et al*., 2000; Warberg *et al*., 2005). However in the present study amplification of target or cockle DNA was not achieved using ITS primers. Amplification of trematode DNA was attempted using the 18Scom region and the small subunit rRNA too. It proved unsuccessful, and it has been shown in previous research that the ITS region is better at determining species than the 18Scom region (Lord *et al*., 2002).

Primers based on previous studies of microsporidians were utilised in the case of *Unikaryon legeri* and partial sequencing was achieved in the present study. These primers had been used in studies of *Dictyocoela* spp. infecting gammarid hosts (Wilkinson *et al*., 2011).

Unikaryon legeri forms spores, which are encased in chitin and protein, which may not have been degraded fully in the extraction process inhibiting DNA recovery. However other studies have successfully extracted DNA from microsporidian spores using commercial kits e.g QIamp tissue kit (Müller *et al*., 1999) and QIamp stool mini kit (Subrungruang *et al*., 2004). Although *M. minutus* do not encyst in the host (Russell-Pinto and Bowers, 1998), they do undergo changes in their tegument when changing from cercariae to

metacercariae in the host; this too could have prevented correct extraction (Bowers and James, 1967).

The utilisation of inappropriate primers may have been the cause of the negative results found. As previously mentioned there is a lack of molecular data concerning the trematode under study and concerning *Unikaryon legeri* and its taxonomy has been revised several times. Failure to amplify fully either of the study species could be related to usage of the incorrect tools. The lack of existing molecular markers led to the usage of general primers that were already in existence. The generic nature of the CO1, 18s, ITS and eukaryotic primers, in combination with possibly interference of other organisms in the DNA, may have affected the subsequent reactions. Cockle DNA, which was not the target species, was repeatedly amplified, which may be due also to the general primers used and the large portion of cockle tissue in comparison to the target species resulting in a dilution effect. It has been suggested that a "cocktail" of primers could be used for species that have proved difficult to amplify (Moszynska *et al*., 2009).

Alternatively, different markers such as microsatellites could be used, however they too require sequence data and none existed in the literature for the species under study. In the present study microsatellites were not utilised due to the amount of time required for development and optimization (Guichoux *et al*., 2011). Despite being labour intensive and costly they are often used for population genetics as microsatellites are highly polymorphic, are good at detecting recent population changes and can be used on closely related species (Guichoux *et al*., 2011). However in recent years the creation of markers has become easier due to next generation sequencing (Guichoux *et al*., 2011).

This study showed variation apparent in populations of cockles and illustrated the differences in DNA quality depending on extraction method. Further investigation of SNPs and disease development and local adaption in bivalves is required, especially those that are commercially exploited is key. In conclusion, this study highlights the lack of molecular data regarding *Meiogymnophallus minutus* and to a lesser extent *Unikaryon legeri*. Despite repeated attempts using several different primers and conditions, correct amplification of either species

DNA proved unsuccessful. Unsuccessful usage of primers utilised in other studies of trematodes may be due to variation in the region primers are created to recognise between species. Further study is undoubtedly warranted to gain more knowledge of these species and creation of more specific primers is a priority before an investigation of any variation in the genetics of widely separated populations can be achieved. In future studies it may be advisable to alter the way trematode metacercariae are recovered, to minimize the amount of cockle tissues included and to use different markers such as microsatellites.

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Even their parasites have parasites.

CHAPTER 8: GENERAL DISCUSSION

DISCUSSION

This thesis examined several aspects of the biology, reproduction, health, and population structure of a range of bivalves in the intertidal area predominantly in soft sediments. Several key findings were made: A. The phenotypic plasticity in marine invertebrate reproductive strategies (Chapter 3 and 4), B. There is a wide range of parasites, and potential bivalve host species in two given areas were identified (Chapter 6), C. The aetiology behind surfacing and i.e. mortalities in cockles is site specific with very specific local causative factors (Chapter 5), D. Parasitized mussels can still be "healthy" and threshold levels of parasites in terms of prevalence and abundance are important in distinguishing between healthy and unhealthy animals (Chapter 6), E. The biology of little known bivalves was investigated (Chapter 4 and 6) and F. Local adaptation in terms single nucleotide polymorphisms in cockles was found but further work is required to screen for cockle and parasite genetics and optimize methodologies (Chapter 7).

Following a 16-month investigation of cockle reproduction, differences between the cockles at the two sites under study were noted and perhaps most importantly after comparing with previous research variation over the last century was noted. Advancement and extension of spawning was seen too following a harsh winter (Chapter 3). Spawning in Ireland was of greater duration and gametogenesis began earlier than has previously been reported in other studies (e.g. Seed and Brown 1977; Navarro *et al*., 1989). It is thought that spawning for longer does not equate to a greater recruitment overall (Dabouineau and Ponsero, 2009) therefore if the trend for milder winters continues the biomass of cockles could be reduced. The possibility of a short-lived species like a cockle experiencing colder winters to allow for greater reproduction is reduced (Cardoso *et al*., 2009). However the current study was performed during two extremely severe winters despite the warming trend – cold winters may be beneficial to cockles as they encourage synchronicity between the sexes. If periodic cold winters are to become the norm then perhaps they will compensate and reset cockle reproductive strategy. Additionally the gonads were assessed by histology in the form of presence/absence and their maturity. Other studies need to be performed to assess if changes are apparent in the amount of gonad and subsequent gametes produced rather than just in the timing of reproductive events.

Variation was clear too in the reproduction of *Mytilus edulis*, this species appeared to respond to alterations in climate perhaps demonstrating the plasticity in its reproductive strategy. Plasticity has been identified as a likely way that animals can cope with alterations in climate through acclimatization potential (Somero 2009; Silvestre *et al*., 2012). As males and females were in synchrony following the cold spell, and there was advancement and extension of the spawning period, indicating that such events may be of benefit to this bivalve. However as in the case of cockle reproduction, timing of gametogenesis and spawning was assessed not the biomass of gonads or gametes, however it has been shown that in *Macoma balthica* egg production was reduced by 7 % only during a mild as opposed to harsh winter (Flach, 2003; Beukema *et al*., 2001; Beukena and Dekker, 2005). Wild mussels are longer lived than cockles (maximum 18-24 years, compared to 9 years in cockles), and it has been suggested that longer lived species will be able to deal with climate variability better, as there is a greater chance of them encountering favorable conditions (Cardoso *et al*., 2009). Despite plasticity observed in *M. edulis* in the present study, the possibility of locally adapted populations of this species developing is low according to a study by Somero (2009) as organisms with planktonic larvae and a wide latitudinal range are less likely to foster such populations.

The population biology of several other common intertidal bivalves was investigated too. It was of interest to study all the bivalves in a soft sediment ecosystem to gain more knowledge of soft sediment bivalves and their biology. . There have been no studies in Ireland previously on *Macoma balthica*, *Angulus tenuis* or *Scrobicularia plana* in this regard. Spawning occurred over winter in *M. balthica* but in spring/summer in *A. tenuis*. Earlier spawning than has been reported in other sites was observed in *M. balthica*. However small sample sizes were used and not every month was sampled so further large-scale studies are needed to investigate their reproductive biology fully with a minimum sample size of thirty each month

One of the most reported incidents in soft sediments in recent years has been that of mass surfacing with subsequent large mortalities in the cockle. Surfaced and buried cockles were compared at two sites over 16 months and the role of several parameters were investigated including age, sex, gonadal maturity and season. The interaction between these parameters and how they and parasites influence

mortality events was considered using multivariate analysis. Site related differences dominated when explaining mortality events. Surfaced cockles from Bannow Bay had higher levels of most parasites recorded, significantly in the case of *Nematopsis* sp. oocysts. In contrast variation between surfaced and buried cockles at Flaxfort Strand appeared to be driven by neoplasia and trematodes. Non-metric multidimensional scaling (NMDS) ordination showed that cockles accumulate parasites until a threshold age of three, and at that age many may die, as the average age of surfaced cockles and the highest prevalence of neoplasia were in cockles of age three. As cockles are known to be able to accumulate high levels of metacercariae with age (Wegeberg and Jensen, 1999) perhaps this age is high risk for neoplasia, which then alters the host-parasite relationship inducing mortality. NMDS ordination allowed for the analysis of a large amount of data across a number of parameters concerning each cockle including health, age, and sex and it allowed for the identification of trends in the data. Wider use of this statistical tool may be valuable, however follow on analysis with ANOSIM showed some of the pitfalls of using this analysis. Due to the large sample size, on occasion the P value showed significance, as it is a function of sample size, while the true measure of difference between factors the Global R did not. However this can be allowed for, once strict adherence to a high Global R is maintained and being aware of what the various parts of the test mean. As NMDS and ANOSIM analysis are rarely used with this kind of data, it is difficult to compare it to other studies. The intensities of parasites was used, but as histology rather than squash preparations were utilized they may be underestimated, and most studies of cockle parasites, trematodes in particular use squash preparations. Histology was the preferred method nonetheless as it allowed for the diagnosis and staging of neoplasia, and to ascertain the sex and reproductive stage of each cockle if gonads were present.

The question was posed can bivalves be parasitized and healthy (Chapter 6). The health of mussels was examined at two sites over a 16-month period. At both sites cockle mortalities have been observed for a number of years but no such mortalities were observed in mussels (i.e. moribund dying animals). Although a range of parasites (e.g. trematodes) and morphological changes were observed in the mussels, they were, in generally low prevalence and were lower than other studies of mussel histopathology (e.g. Bignell *et al*., 2008; Bignell *et al*., 2011).

However parasite burden may have been underestimated as histology was used – unlike squash preparations a section of tissue is screened not the whole body. There was variation between the two sites in terms of parasite fauna, prevalence and intensity, which may be due to the presence of other hosts in the area. There was a significantly higher level of the three trematode species identified in Flaxfort Strand rather than Bannow Bay, however from Chapter 4 it can be seen that mussels from Flaxfort Strand were significantly greater in size and spawned twice compared to once in Bannow Bay. Therefore perhaps it could be concluded that despite being parasitized they are continuing to spawn and grow so are they truly unhealthy. Other factors such as metabolic age could be taken into account also as a measure of health and fitness too rather than just parasitism.

A parasite atlas was compiled, showing the range, prevalence, and intensity of all the parasites in the bivalves under study at both sites. Interactions between these various bivalves in the sediment may occur as they are suspension feeders exploiting plankton, and fish and birds predate them upon. The ubiquity of parasites, digeneans in particular was emphasized as they were recorded in all bivalves examined except *Mya arenaria*. One pathological condition, disseminated neoplasia, in addition to 22 parasite taxa were identified from *Cerastoderma edule*, *Mytilus edulis*, *Macoma balthica*, *Angulus tenuis*, *Scrobicularia plana* and *Ruditapes* sp. The parasite fauna was dominated by digenean trematodes, in addition to ciliates. The greatest range of parasites was identified in cockles and mussels. This may be due to their wide distribution, greater size compared to the other species, and accessibility to other hosts (on top of the sediment) and infective stages as well as potentially to the larger sample sizes collected as they were the main focus of the studies. The cockle is particularly striking as a host species to such a wide range and number of parasites and pathologies. Some parasites had a wide range of potential hosts e.g. *Nematopsis* sp. oocysts while others such as *Meiogymnophallus minutus* were restricted to *S. plana* and *C. edule*. The wide range of parasites identified and across several hosts emphasizes the diversity that parasites represent, and the impact they may have influencing the host population and therefore the community as a whole. Parasites can alter sex ratios, fecundity, growth, and enhance mortality therefore they may influence the host and the community at

many levels (Poulin, 1999). Therefore, including parasites in any study of the intertidal is important due to their biomass and the influence that they exert in driving ecosystem change. In the current study however the limitations of the chosen technique – histology may have allowed for an underestimation of parasite numbers. Using histology also it is particularly difficult to identify trematode species accurately unlike using squash preparations. Therefore species could not be identified accurately , and although the method used to ascertain intensity of parasites between bivalves was comparable across this study (as all were treated the same) as previously mentioned many studies use squash preparations so comparison to other literature is difficult. Attempts were made to investigate the population structure of *Cerastoderma edule*, *Meiogymnophallus minutus* and *Unikaryon legeri* across Europe using molecular means. Several molecular markers were used (CO1, ITS) but successful amplification of cockle DNA was achieved only using 18s markers, and Qiagen extracted DNA. A small nucleotide polymorphism was found in cockles from Cork, Ireland, which may be indicative of local adaptation compared to Moroccan and Genbank sequences. The inability to amplify trematode or microsporidian DNA successfully may have been due to the non-specific primers used. The paucity of molecular studies concentrating on both trematodes and microsporidia meant that more specific primers were not available. Further studies of trematodes and microsporidia using molecular means are warranted.

Overall the study contributed to the knowledge of soft sediment bivalves, their health and aspects of their physiology and looks at some of the external drivers of disease development and mortality events.

MAIN FINDINGS

- Reproduction in *Cerastoderma edule* has altered over the course of the last century (Chapter 3) perhaps as a consequence of climate change.
- *Mytilus edulis* reproductive strategy can respond rapidly to external factors, with climate variability impacting on this process (Chapter 4).
- The causes of surfacing and mortalities in cockles are site specific, and there appears to be a threshold age of three at which a large portion die (Chapter 5).
- A threshold level of infection is required and despite being parasitized mussels should not be considered unhealthy as they continue to spawn and grow (Chapter 6).
- Across a range of taxa in one ecosystem there is high levels of parasite diversity, some bivalves support a greater range of parasites, and parasites may be host specific.The reproductive biology of *Macoma balthica* and *Angulus tenuis* was documented in Ireland for the first time, and their parasite fauna in addition to that of *Scrobicularia plana* was examined (Chapter 4 and 6).
- Local adaptation was apparent in cockles from Flaxfort Strand, Cork, Ireland, as they had a small nucleotide polymorphism compared to Moroccan and Genbank sequences (Chapter 7).

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Just thanks to everyone for putting up with me while I babbled about statistics and cockles for 3 years.

APPENDIX 1

Table: CO1 mastermix a and thermocycling conditions.

CO1 Mastermix a for 1 Individual		**Thermocycling Conditions as per Królová-Hromadová *et al*., 2001**			
Substance	Quantity (µl)	Stage	Temperature (° C)	Time (s)	Cycles
5x Buffer	5	1	94	300	1
dNTPs	5				
Forward	0.1	2	94	60	30
Reverse	0.1		50	60	30
Taq	0.1		72	120	30
ddH2O	13.7				
DNA	1	3	72	300	1

APPENDIX 2

Table: CO1 b mastermix and thermocycling conditions.

CO1 Mastermix b for 1 Individual		**Thermocycling Conditions as per Królová-Hromadová *et al*., 2001**			
Substanc e	Quantity (µl)	Stage	Temperature (° C)	Time (s)	Cyc les
5x Buffer	10	1	94	300	1
dNTPs	10				
Forward	0.2	2	94	60	30
Reverse	0.2		50	60	30
Taq	0.2		72	120	30
$MgCl_2$	10				
ddH2O	27.4	3	72	300	1
DNA	2				

APPENDIX 3

Table: CO1 c mastermix and thermocycling conditions.

CO1 Mastermix c for 1 Individual		**Thermocycling Conditions Modified from Zhang *et al*., 2005**			
Substance	Quantity (µl)	Stage	Temperature (° C)	Time (s)	Cycles
5x Buffer	5	1	95	60	1
dNTPs	5				
Forward	0.25	2	94	20	35
Reverse	0.25		56	30	35
Taq	0.1		72	30	35
$MgCl_2$	0.5				
ddH2O	12.9	3	72	420	1
DNA	1				

APPENDIX 4

Table: 18Scom mastermix and thermocycling conditions.

18Scom Mastermix		**Thermocycling Conditions modified from Zhang *et al*., 2005**			
Substance	Quanitity (µl)	Stage	Temperature (° C)	Time (s)	Cycles
5x Buffer	5	1	95	60	1
dNTPs	5				
Forward	0.25	2	94	20	35
Reverse	0.25		56	30	35
Taq	0.1		72	30	35
$MgCl_2$	0.5				
ddH2O	12.9	3	72	420	1
DNA	1				

APPENDIX 5

Table: Eukaryote mastermix and thermocycling conditions.

Eukaryote Mastermix		**Thermocycling Conditions Medlin *et al.*, 1988**			
Substance	Quanitity (µl)	Stage	Temperature (° C)	Time (s)	Cycles
5x Buffer	5	1	95	300	1
dNTPs	5				
Forward	1	2	65	180	40
Reverse	1		90	90	40
Taq	1.25				
$MgCl_2$	1.5				
ddH2O	9.25				
DNA	1				

APPENDIX 6

Table: ITS Region mastermix and thermocycling conditions.

ITS Region Mastermix		**Thermocyling Conditions Warberg *et al*., 2005**			
Substance	Quantity (µl)	Stage	Temperature (° C)	Time (s)	Cycles
5x Buffer	4	1	94	180	1
dNTPs	3.2				
Forward	0.1	2	94	30	40
Reverse	0.1		55	40	40
Taq	0.1		72	60	40
ddH2O	9.5				
DNA	3	3	72	420	1

APPENDIX 7

Table: *Unikaryon legeri* mastermix and thermocycling conditions.

***Unikaryon legeri* Mastermix**		**Thermocycling Conditions from Wilkinson *et al*., 2011**			
Substance	Quanitity (µl)	Stage	Temperature (° C)	Time (s)	Cycles
5x Buffer	2.5	1	95	300	1
dNTPs	1				
Forward	1	2	35	60	35
Reverse	1		50	60	35
Taq	0.25		72	60	35
MgCl2	1.25				
ddH2O	17	3	72	420	1
DNA	1				

APPENDIX 8: Oral presentations, posters and publications.

Department of Biological, Earth and Environmental Sciences & SUSFISH Related Oral Presentations:

Morgan, E., (2010). The influence of climate change on soft sediment bivalves and their pathogens. **Plans and Progress Talk, School of BEES, Cork, Ireland.**

Morgan, E., (2010). The influence of climate change on soft sediment bivalves and their pathogens. **School of BEES Graduate Studies Committee, Cork, Ireland.**

Morgan, E., (2010). The influence of climate change on soft sediment bivalves and their pathogens. **Maths Modelling Workshop, Cork.**

Morgan, E., O'Riordan, R.M., Culloty, S.C., (2011). The influence of climate change on soft sediment bivalves and their pathogens. **SUSFISH Progress Meeting, Swansea, Wales.**

Morgan, E., O'Riordan, R.M., Culloty, S.C., (2011). The influence of climate change on soft sediment bivalves and their pathogens. **SUSFISH Progress Meeting, Aberystwyth, Wales.**

Morgan, E., O'Riordan, R.M., Culloty, S.C., (2012). The influence of climate change on soft sediment bivalves and their pathogens. **SUSFISH Progress Meeting, Cork, Ireland.**

Morgan, E., O'Riordan, R.M., Culloty, S.C., (2012). The influence of climate change on soft sediment bivalves and their pathogens. **SUSFISH Progress Meeting, Swansea, Wales.**

Conference Oral Presentations:

Morgan, E., O'Riordan, R.M., Culloty, S.C., (2010). The influence of disseminated neoplasia and trematode infections on summer mortality in the cockle, *Cerastoderma edule*. **European Marine Biological Synposium, Edinburgh, Scotland**.

Morgan, E., Lynch, S.A., O'Riordan, R.M., Culloty, S.C., (2012). Comparison of the health status of the common cockle, *Cerastoderma edule* at two sites in Southern Ireland. **National Shellfisheries Association, Seattle, America.**

Morgan, E., Lynch, S.A., O'Riordan, R.M., Culloty, S.C., (2012). Health status of the common cockle, *Cerastoderma edule* in Ireland. **Marine Biological Association Post Graduate Conference, Cork, Ireland**.

Morgan, E., O'Riordan, R.M., Culloty, S.C., (2012). Aspects of the biology and pathology of *Cerastoderma edule* in the Irish Sea. **Environmental Research Insitute Open Day, UCC.**

Poster Presentations:

Morgan, E., Cross, M.E., Lynch, S.A., O'Riordan, R.M., Culloty, S.C., (2011). SUSFISH productivity in the Irish Sea: Working towards a sustainable future. (SUSFISH). **National Shellfisheries Association, Baltimore, America**.

Morgan, E., O'Riordan, R.M., Culloty, S.C., (2011). Aspects of the Biology and Pathology of the Cockle, *Cerastoderma edule* in the Irish Sea**. National Shellfisheries Association, Baltimore, America**.

Morgan, E., O'Riordan, R.M., Culloty, S.C., (2012). Aspects of the Biology and Pathology of the Cockle, *Cerastoderma edule* in the Irish Sea. **Environmental Research Insitute Open Day, UCC.**

Publications:

Morgan, E., O'Riordan, R.M., Kelly, T.C., Culloty, S.C., (2012). The influence of disseminated neoplasia, trematode infections and gametogenesis on surfacing and mortality in the cockle, *Cerastoderma edule*. *Diseases of Aquatic Organisms* **98**:73-84.

Morgan, E., O'Riordan, R.M., Culloty, S.C., (2012). Climate change impacts on potential recruitment in an ecosystem engineer. *Ecology and Evolution*, In press.

Morgan, E., Lynch, S.A., O'Riordan, R.M., Culloty, S.C., (2012). Plasticity in mollusc reproduction: identifying drivers of variation in *Mytilus edulis*. PLOS ONE, In review).