Biotic and abiotic factors influencing haplosporidian species distribution in the cockle *Cerastoderma edule* in Ireland

S. Albuixech-Martí [a,*], S.A. Lynch [a,b], S.C. Culloty [a,b,c]

[a] *School of Biological, Earth & Environmental Sciences,* [b] *Aquaculture & Fisheries Development Centre, Environmental Research Institute, and* [c] *MaREI Centre for Marine and Renewable Energy, Environmental Research Institute, University College Cork, The Cooperage, Distillery Fields, North Mall, Cork, Ireland.*

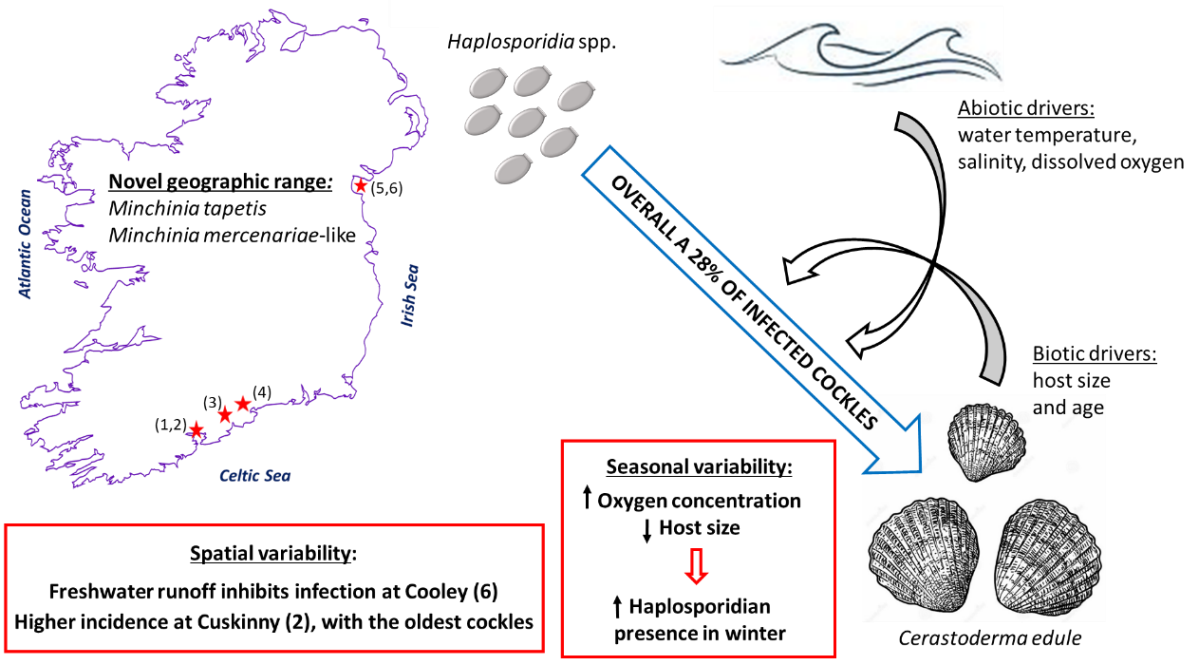
*Corresponding author:
Sara Albuixech-Martí,
School of BEES, The Cooperage
Distillery Fields, North Mall, Cork, Ireland.
Telephone: +353 (0)892193850
Email: saraalbuixechmarti@ucc.ie

Abstract

The Phylum Haplosporidia consists of four genera (*Minchinia*, *Haplosporidium*, *Urosporidium* and *Bonamia*) that are endoparasitic protists of a wide range of marine invertebrates including commercial bivalve species. Characterization of haplosporidian species remains a challenge due to their patchy spatial and temporal distributions, host-restricted occurrence, and poorly known life cycles. However, they are commonly associated with significant mortality events in bivalves. Due to the recent sporadic mortality events that have occurred in cockles in Europe, the objectives of this study were to determine the diversity, distribution and seasonality of haplosporidian species in *Cerastoderma edule* populations at several Irish sites. The role of abiotic (temperature, salinity and dissolved oxygen in water) and biotic (cockle size and age) factors as drivers or inhibitors of haplosporidian infection were also assessed. Cockles (n=998) from the intertidal were sampled from April/July 2018 to April 2019 at three sites with no commercial fishing activity on the south coast (Celtic Sea) and one site on the northeast coast (Irish Sea) with an active commercial fishery. Screening of the cockles by molecular techniques (PCR, Sanger sequencing) and by histopathology was carried out. Two species were identified and confirmed in Irish *C. edule* for the first time, *Minchinia mercenariae*-like (14.8%) and *Minchinia tapetis* (29.6%). Similar to other haplosporidian parasites, the *Minchinia* spp. detected in our study were present year-round at all sites, except for *M. tapetis* in Youghal Bay (Celtic Sea). Coinfection of both *Minchinia* species was only observed in Cork Harbour (Celtic Sea) and Dundalk Bay (Irish Sea), where *Minchinia* spp. showed a higher presence compared to Youghal Bay and Dungarvan Harbour (Celtic Sea). Moreover, haplosporidians detected with generic primers, were present at all of the sample sites throughout the year but had a higher occurrence during the winter months and were positively correlated with dissolved oxygen. Likewise, smaller and older *C.edule* seemed to be more vulnerable to the haplosporidian infection. Furthermore, haplosporidian distribution displayed spatial variability between and within sample sites, with the highest presence being observed in cockles at one of the commercially fished Dundalk beds, while the lowest presence was observed in cockles at the second Dundalk bed that was more influenced by freshwater runoff when the tide was out. Findings from this study provide additional information on the distribution and seasonal presence of novel haplosporidian species and their potential abiotic and biotic drivers/inhibitors of infection.

Keywords: cockle health, Haplosporidia, *Minchinia*, coinfection, ecological drivers.

Graphical abstract:



Highlights:

- ✓ *Minchinia tapetis* and *M. mercenariae*-like were identified in Irish *C.edule*.
- ✓ Coinfection of both *Minchinia* spp. was detected in Irish *C.edule.*
- ✓ Haplosporidians showed spatial and seasonal variability with higher winter presence.
- ✓ *Haplosporidia* spp. was related positively to dissolved oxygen in the water.
- ✓ Lower presence of *Haplosporidia* spp. at low salinity sites.
- ✓ Smaller and older *C.edule* were more vulnerable to the infection.

1. Introduction

Cockles (family Cardiidae), including the common cockle *Cerastoderma edule,* are bivalves that occur intertidally and subtidally in estuaries and sandy bays around the European coastline (Malham et al. 2012, Longshaw and Malham, 2013). In general, cockles have been well-studied and there is evidence that they are responsible for different ecosystem services, i.e. carbon storage, energy cycling, food source for seabirds, etc. (Magalhães et al. 2017). They are also good sentinel and bioindicator species (Malham et al., 2012), and are commercially exploited with a high economic value (Rowley et al., 2014). There are two European cockle species that are morphologically similar and that sometimes coexist (Malham et al. 2012). The edible cockle *Cerastoderma edule* (Linnaeus, 1758) is one of the most common infaunal bivalve species in more northern European soft-sediment coastal waters and is commonly found on Irish coasts (Fermer et al. 2011, Morgan et al. 2012). The lagoon cockle *Cerastoderma glaucum* (Poiret, 1789), is considered a more southern European species, coexisting with *C. edule* in several localities of Portugal, France, UK and NW Spain (Malham et al. 2012; Carballal et al. 2016).

Cockles are hosts to a wide range of parasites, pathogens and commensals, directly affecting individual cockle health and cockle population dynamics (Longshaw and Malham, 2013). Cockles may be host to multiple macro-parasite infections, particularly trematodes (de Montaudouin et al., 2000), but may also be infected by a range of micro-parasites including protists (Coen and Bishop, 2015). One protistan pathogen group that cockles are susceptible to are the Haplosporidia, which are widespread and cause significant losses (Longshaw and Malham, 2013). At present there are thirty-six recognised species in the phylum Haplosporidia belonging to four genera, *Urosporidium* parasitizing trematodes or turbellaria infecting clams, cockles, oysters, and swimming crabs (Perkins, 1979; Carballal et al., 2005), *Minchinia* parasitizing clams, mussels and cockles (Azevedo, 2001; Ford et al., 2009; Lynch et al., 2014; Ramilo et al. 2018; Lynch et al., 2020), *Haplosporidium* being major pathogens of concern in oysters (Burreson and Ford, 2004) but have also been detected in cockles (Azevedo et al., 2003; Engelsma et al., 2011), and *Bonamia* in also being serious pathogens of oysters (Culloty and Mulcahy, 1996; Engelsma et al., 2014). Characterising the diversity and distribution of these parasites remains a challenge due to their patchy spatial and temporal distributions, host-restricted occurrence, and poorly known life cycles (Hartikainen et al., 2014). Detection of haplosporidian taxa is hindered by the use of broadly targeted molecular probes that are unsuitable for the highly divergent genes that characterise parasite groups (Burreson and Ford,

2004; Hartikainen et al., 2014). Despite that, numerous novel haplosporidians continue to be reported, although not specifically identified, from many different invertebrate hosts and habitats, highlighting the possibility that the geographic range of haplosporidian species is significantly greater than originally documented (Burreson and Ford, 2004; Lynch et al., 2013; Lynch et al., 2014; Arzul and Carnegie, 2015; Ramilo et al., 2018; Lynch et al., 2020).

A number of studies have reported the detection of *Minchinia* spp. in cockles although several clam species have been described as their natural hosts. *Minchinia mercenariae* was first described in the hard clam *Mercenaria mercenaria* along the eastern United States coastline (Ford et al., 2009). *Minchinia tapetis* was reported in clams such as the grooved carpet clam/Palourde clam *Ruditapes decussatus*, the pullet carpet clam *Venerupis corrugata* and the banded carpet clam *Polititapes rhomboides* from Galicia, NW Spain (Villalba, et al. 1993) and in *R. decussatus* and golden carpet clam *Polititapes aureus* from Huelva coast, SW Spain (Navas et al. 1992); as well as in *R. decussatus* from Portugal (Azevedo, 2001). *Minchinia mercenariae* and *Minchinia tapetis* have been reported to cause infections in cockles *Cerastoderma edule* in Netherlands and UK, where it was implicated as the cause of host population crashes (Engelsma et al., 2011; Longshaw and Malham, 2013). A *Minchinia mercenariae*-like parasite was recently detected for the first time in *Cerastoderma edule* on the east and south coasts of Ireland (Lynch et al., 2020) and in *C. edule* in Spain (Ramilo et al. 2018).

The immune-competence relationship between parasite and host is influenced by environmental conditions and stressors, which may compromise the immune response and the balance in host-pathogen interactions, causing disease or even mortality if the host resistance is overcome (Selakovic et al. 2014). Environmental conditions potentially influence disease processes through differential effects on pathogens and hosts (Lafferty and Holt, 2003). Moreover, parasites may reduce the tolerance threshold of host organisms to extreme environmental conditions such as oxygen depletion, extreme temperatures and salinity fluctuations (Longshaw and Malham, 2013). Consequently, parasite burdens that are considered harmless in favourable environmental conditions could become stressful harmful agents in unfavourable environmental conditions (Longshaw and Malham, 2013).

For host-parasite systems in estuarine and marine environments, abiotic factors (e.g., temperature, salinity, dissolved oxygen, etc.) are particularly critical in shaping the host-parasite interactions (Coen and Bishop, 2015). Temperature is one of the most important factors

determining physiological processes, ecological patterns and the limits on the geographic distribution of bivalves (Verdelhos et al., 2015a). Thermal stress is, therefore, a critical environmental impacting factor, especially in intertidal habitats more exposed to temperature changes (Helmuth et al., 2010). Salinity is a major factor impacting estuarine bivalves, which are often exposed to short-term (tidal) and long-term (rain periods) changes in salinity, promoting a physiologically stressful environment, especially in cases of abrupt salinity changes (Verdelhos et al., 2015b). Hypoxia or low dissolved oxygen is also a persistent problem in estuaries and nearshore coastal ecosystems (Breitburg et al., 2015). Low dissolved oxygen stress can lead to increased susceptibility to disease (Breitburg et al., 2015) and, consequently, to sublethal and lethal effects on many species, including molluscs (Stickle et al., 1989). Variation in the physical environment may therefore alter the host-pathogen interactions and affect both spatial and temporal patterns of economically and ecologically important species and the severity of epizootics (Breitburg et al., 2015).

Besides climatic stressors, biotic factors may also have a key role in shaping host-parasite interactions. Former authors have demonstrated that age can affect organism response to stress (Taskinen and Saarinen, 1999; Renault and Novoa, 2004; Clark et al., 2013). Moreover, older hosts are more likely to accumulate disease-causing pathogen agents over time, as the duration of exposure to parasites is longer than in younger hosts (Lafferty and Kuris, 2009). Likewise, the infection success is likely to be higher in a population of larger cockles due to a much larger filtration capacity and, consequently, higher intake rate of parasites (Mouritsen et al., 2003). These findings highlight the need to consider biotic factors (size and age class) as potential drivers or inhibitors of infection.

The objectives of this study were to determine (i) the diversity, (ii) spatial distribution and (iii) seasonality of haplosporidian species in Irish *Cerastoderma edule* populations along the coastline of the Irish and Celtic Seas and to better understand (iv) the abiotic (seawater temperature, salinity and dissolved oxygen in water) and biotic (cockle body size and age) drivers or inhibitors of infection. We hypothesise that the expression of haplosporidian disease is driven by biological and environmental factors and, therefore, varies spatial and seasonally along Irish coasts. Our study extended the understanding of how the physical environment, as well as the host susceptibility, can modulate disease processes in aquatic environments by linking spatial patterns and seasonality of parasite occurrence to relevant environmental parameters (temperature, salinity and dissolved oxygen in water) and biotic factors (cockle size and age).

2. Material and Methods:

2.1. *Sampling:*

Cockles were sampled (from April/July 2018 to April 2019) within the commercial fishery at Dundalk Bay (Annagassan and Cooley) on the northeast coast (Irish Sea) and from Dungarvan Harbour, Youghal Bay and Cork Harbour (Ringaskiddy and Cuskinny) on the south coast (Celtic Sea) of Ireland where there is no commercial fishing activity (Figure 1, Table 1). The six locations are Special Protection Areas (SPAs) under the E.U. Birds and Habitats Directives, providing good quality feeding areas and roost sites for an excellent diversity of waterfowl species (www.npws.ie/protected-sites/spa).

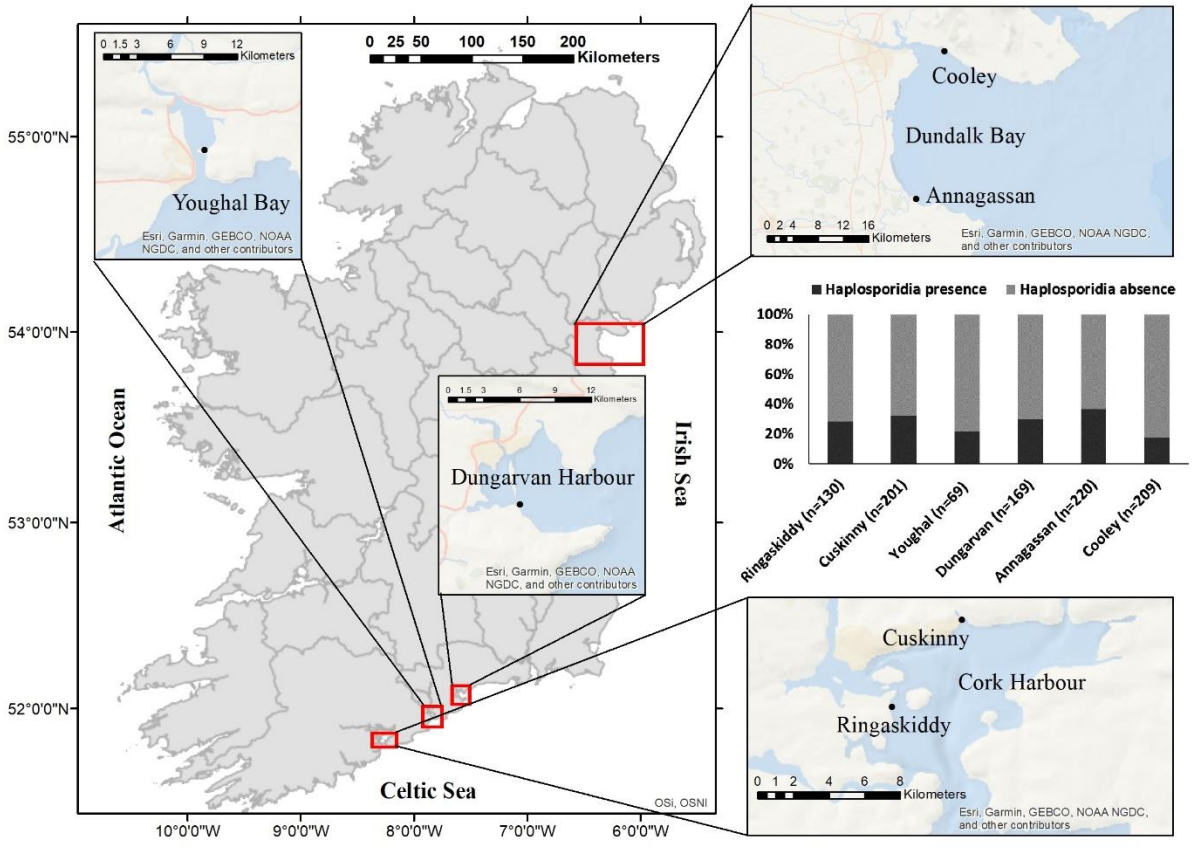


Figure 1: Map of Ireland highlighting the cockle sample sites and the mean haplosporidian presence and absence (%) at each sample site (Ringaskiddy and Cuskinny in Cork Harbour; Youghal Bay; Dungarvan Harbour; Annagassan, and Cooley in Dundalk Bay).

Table 1: Sample site description (www.npws.ie/protectedsites/spa) including water quality rating (EPA, 2017) and anthropogenic activities.

Sites		Coordinates	Trophic status	Description
Cork Harbour	**Ringaskiddy**	51°49'52.11"N 8°17'56.64"W	Unpolluted	A large, sheltered bay system, with several river estuaries and intertidal mudflats. Ringaskiddy is located near to an important industrial area and is a busy ferry/shipping terminal; Cuskinny is a Marsh Nature Reserve supporting an array of habitats within a relatively small area, for a diverse community of wildlife.
	Cuskinny	51°51'29.94"N 8°15'49.50"W		
Youghal Bay		51°57'18.52"N 7°50'3.51"W	Intermediate polluted	A moderately-sized, sheltered south-facing estuary, with intertidal sediments, mostly consisting of mud or sandy mud.
Dungarvan Harbour		52° 3'52.38"N 7°36'10.37"W	Unpolluted	A marine habitat with the inner bay being extremely sheltered and with extensive mud and sand flats. Extensive farming of Pacific oysters *Crassostrea gigas* in this bay.
Dundalk Bay	**Annagassan**	53°52'52.30"N 6°20'7.77"W	Unpolluted	An open shallow bay with extensive salt marshes and intertidal sand and mudflats. The main cockle fishery in Ireland (Fahy et al. 2005). Annagassan and Cooley are oppositely located, with Cooley mainly affected by freshwater runoff from Castletown River and Flurry River due to its proximity.
	Cooley	54° 0'16.84"N 6°17'43.17"W	Intermediate polluted	

Cockles of all available sizes were collected by hand raking from the intertidal area of each site at spring tide (0.5-1m) and at monthly intervals from April/July 2018 to April 2019. The sample size was dependant on the available abundance at each site and season (Table 2). The cockles were collected from the surface and within the sediment at relatively shallow depths (<15 cm), hence a differentiation was not considered between them. Cockles were kept at ambient temperature during the fieldwork and, once back at the laboratory, were processed either on the same day or were held overnight at 4°C and were processed the next day.

Table 2: Sample sites, dates and number of collected cockles.

Sampling dates	Cork Harbour - Ringaskiddy	Cork Harbour - Cuskinny	Youghal Bay	Dungarvan Harbour	Dundalk Bay- Annagassan	Dundalk Bay- Cooley
April 2018	23	28	-	-	-	-
June 2018	19	57	-	-	-	-
July 2018	-	-	11	51	60	58
August 2018	12	27	-	-	39	31
October 2018	22	30	-	-	30	30
November 2018	-	-	16	58	-	-
December 2018	9	6	-	-	31	30
February 2019	15	23	30	30	30	30
April 2019	30	30	12	30	30	30
Totals	**130**	**201**	**69**	**169**	**220**	**209**

(-) No samples

2.2. *Sample processing and diagnostic methods:*

2.2.1. Morphometrics:

Measurements included (A) cockle body size (height, length and width (mm)) that were recorded using Vernier callipers, as well as (B) the wet weight (g) of each individual, after drying the excess of water from the shell, using an electronic balance scale. The (C) cockle's age was determined by counting the annual winter growth rings. For verification and validation purposes, the number of growth rings was confirmed by a second researcher, as it is recognised that variations in growth during the same year lead to the possible formation of extra stria (de Montaudouin, 1996).

2.2.2. Histology:

A transverse section of each cockle, including the digestive gland, gonads, gills and mantle tissues, was excised, as well as a section of the hinge ligament and a section of the tip of the foot for histological examination. The three sections were placed in a labelled histocassette and were preserved in Davidson's fixative for 48 h (Shaw and Battle, 1957) prior to processing. Paraffin-embedded tissue blocks were sectioned at 5μm and stained using haematoxylin and eosin (Howard and Smith, 1983). Tissue slides were screened at a magnification of 40x and under oil at 100x for visualisation of the parasite.

2.2.3. Molecular techniques for cockle species identification and haplosporidian species screening:

A small piece of gill tissue (2-5 mm^2 of tissue) from each cockle was taken and stored at -20°C for molecular assays, to determine the cockle species and to screen for haplosporidian species. Genomic DNA from the cockle gill tissue was extracted using the chelex-100 extraction method (Walsh et al., 1991; Lynch et al., 2008).

To determine the cockle species present, PCR was carried out to amplify the nuclear DNA markers ITS-for / ITS Ce-R / ITS Cg-R to differentiate between the presence of *Cerastoderma edule*, *Cerastoderma glaucum* species or hybrids (Freire et al. 2011). Amplification was conducted in 20µl of the reaction mixture containing 4µl of 5x green buffer, 4µl of dNTPs (1.25mM), 0.2µl of each primer (ITS-for / ITS Ce-R / ITS Cg-R, Table 3), 0.1µl of GoTaq polymerases, 10.3µl of ddH_2O and 1 µl of total genomic DNA under the following conditions: 95°C for 3 minutes (1 cycle), 95°C for 30s, 56°C for 30s, 72°C for 30s (35 cycles) and 72°c for 5 minutes. Electrophoresis of the amplification products was conducted in a 2% agarose gel. The expected amplified product (amplicon) size for *C. edule* was 190bp, and for *C. glaucum* 470bp and both bands would occur simultaneously in hybrids (Freire et al. 2011).

PCR screening for haplosporidian detection in cockle DNA (n = 998) was carried out using generic haplosporidian HAP-F1 and HAP-R3 primers that amplify small regions of the SSU rDNA of most haplosporidians (Renault et al., 2000; Molloy et al., 2012, Table 3). The amplification was conducted in 20µl of the reaction mixture containing 4µl of 5x green buffer, 4µl of dNTPs (1.25mM), 0.4µl of each primer (Table 3), 0.2µl of GoTaq polymerases, 10µl of ddH_2O and 1 µl of total genomic DNA. The reaction mixtures were cycled in a thermal cycler with an initial cycle of 95°C for 3 minutes, 35 cycles at 95°C for 30s, 48°C for 30s, and 72°C for 30s with final extension at 72°C for 5 minutes. Electrophoresis of the amplification products was conducted in a 2% agarose gel.

2.2.4. Primer design and optimisation of specific PCRs for *Minchinia tapetis* and *Minchinia mercenariae*-like:

Direct sequencing was carried out on representative PCR products amplified using generic haplosporidian HAP-F1 and HAP-R3 primers (Renault et al., 2000; Molloy et al., 2012) to identify the species present in the samples. Genomic DNA from three selected individuals

from each sample site (n = 18) was isolated and purified using the QIAquick Gel Extraction Kit (QIAgen) prior to direct sequencing. Both the forward and reverse strands of DNA samples were sequenced commercially by Eurofins Genomics (Germany).

The genomic DNA sequences obtained from the sequenced samples were analysed by BLAST (Basic Local Alignment Search Tool) and were aligned by Clustal Omega (Multiple Sequence Alignment) along with the sequences deposited in GenBank (NCBI) for both identified species, *Minchinia tapetis* (KF208603.1, Hartikainen et al., 2014) and *Minchinia mercenariae* (FJ518816.1, Ford et al., 2009), to distinguish regions of the primary sequence unique to each of the identified species, which would be good candidates for specific PCR primers. Subsequently, Primer3Web version 4.1.0 and OligoCalc 2007 (Oligonucleotide Properties Calculator) were used to design the suitable specific PCR primers based on those regions. Each of the candidate forward and reverse PCR primers (Table 3) were tested in the appropriate pairings and in separate PCR reactions with genomic DNA from *Cerastoderma edule* that were positive with the HAP-F1/HAP-R3 primers (n = 277) (Table 4). Optimised reaction conditions in both PCRs were the same as described above for the PCR using HAP-F1/HAP-R3 primers, except for the quantity of each primer, that was 0.2µl, and the annealing temperature that was 60°C. From all the PCR products amplified using each pair of primers a random subsample (n = 12 from *Minchinia tapetis* PCR and n = 12 from *Minchinia mercenariae*-like PCR) was selected to be purified (by QIAquick Gel Extraction Kit) and sequenced commercially to confirm the species identity of the resulting outcomes and the validity of our primers. In addition, a random subsample (n = 5) among the negative samples for *Minchinia*-specific screening was purified (by QIAquick Gel Extraction Kit) and sequenced commercially to detect the presence of other haplosporidian species occurring in the samples not detected by the newly designed primers.

Table 3: Description of PCR primer pairs used, showing sequences for each forward and reverse primer and expected product size.

Primer pair	Primer sequence (5'–3')		Product size (bp)	Primer specificity
	Forward	Reverse		
ITS-for ITS Ce-R	GTT TCC GTA GGT GAA CCT G	AAG CAG CGA GAA GCC GTT C	190	*Cerastoderma edule*
ITS-for ITS Cg-R	GTT TCC GTA GGT GAA CCT G	AAT TCG CCA TCG TCG G	470	*Cerastoderma glaucum*
HAP-F1 HAP-R3	GTT CTT TCW TGA TTC TAT GMA	AKR HRT TCC TWG TTC AAG AYG A	Variable	*Haplosporidia* spp.
TAP-For TAP-Rev	ATC TAA CTA GCT GTC GCT AAC TCG T	CTT TCA AGA TTA CCC GGC TCT GC	165	*Minchinia tapetis*
MER-For MER-Rev	ATC TAA CTA GCT GTC ACT ATG GAA AA	ACG CAC ATT AAA GAT TGC CCA GCT CTT T	170	*Minchinia mercenariae*-like

2.3. *Environmental data:*

This study was conducted using E.U. Copernicus Marine Service Information (marine.copernicus.eu/services-portfolio/access-to-products). Three relevant environmental parameters critical in shaping host-parasite interactions in estuarine and marine environments (Coen and Bishop, 2015) were selected for this study: seawater temperature, salinity and dissolved oxygen in water. Recorded data in seawater temperature (°C), salinity (PSU) and dissolved oxygen (mmol/m^3), taken monthly at a depth of 0.5m below sea level, were downloaded by ArcGIS Desktop 10.5.1 Redlands, CA (Environmental Systems Research Institute, 2017) throughout the duration of the study (from April 2018 to May 2019) from the different sample sites (coordinate-based) within the Irish and Celtic Seas.

2.4. *Statistical analysis:*

Statistical analysis was performed in R version 3.2.3. statistical software. The haplosporidian occurrence (based on the generic haplosporidian PCR using HAP-F1/HAP-R3 primers) was modelled as parasite presence/absence in each individual cockle and used as the response variable in the subsequent analysis to examine the spatial and seasonal variability and the effects of the biological and environmental variables on the parasite distribution.

A Generalised Linear Mixed Model (GLMM), following binomial distributions for presence/absence data (lme4 package; Bates et al., 2015), was conducted including our Sites and Seasons as fixed terms and the Nested Sites as a random term (Full_Model), i.e. the sample sites were nested in the different bays, as some of the sites are not totally independent since they are in the same bay. Subsequently, a binomial GLMM was performed considering only the Seasons as a fixed term and the Nested Sites as a random term (Sites_Model) to determine how much variability in parasite presence depended on spatial scale when this model was compared to the Full_Model. Likewise, a binomial GLMM was conducted considering only the Sites as a fixed term and the Nested Sites as a random term (Seasons_Model) to assess the seasonal effect on haplosporidian presence when this model was compared to the Full_Model. Both reduced models were compared to the Full_Model using likelihood ratio tests following Chi-square distributions to determine whether the presence of parasites observed were significantly different ($p(Chisq)<0.05$) among the sample sites and throughout the year. Visual inspection of residual plots and model validation with each above model revealed that the model assumptions were satisfied in all the cases.

Collinearity between the different biotic and abiotic variables was examined using non-parametric Kendall's Rank Correlation Tau (τ), displaying a strong correlation ($\tau > 0.8$) between the different cockle size measurements taken (cockle wet weight, height, length and width). Consequently, only cockle height was selected as a factor to be analysed in the consecutive modelling. Moreover, all the explanatory variables were standardized (x-mean/SD) to improve comparisons in the models.

Subsequently, a series of binomial GLMMs were conducted to investigate the relative importance of the biotic and abiotic factors in determining the presence of host infections in the field. Each GLMM included parasite presence/absence as the response variable, none (null model) or a single explanatory variable (1-factor models) as the fixed effect and Nested Sites as well as Seasons as random terms. Competing models were compared based on the Akaike

Information Criterion (AIC), with an efficient model being one that explains a relatively large amount of the variance in the response variable, using relatively few independent variables to do so (MuMIn package; Barton, 2018). A lower AIC value indicates a better fit of the model to the data, thus, the models with the lowest AIC scores were selected as the best performing driver models. Visual inspection of residual plots and model validation with each above model revealed that the model assumptions were satisfied in all the cases.

Then, 2-factor models (binomial GLMMs) were produced with two fixed effects -the top performing driver plus each of the other explanatory variables in turn- also considering Nested Sites and Seasons as random terms; and including an intercept-only model (null model) against which every other model was compared. Again, the best performing models were chosen based on the lowest AICs and the forward selection procedure was terminated at this point to avoid overfitting of the data (Goedknegt et al., 2019). Visual inspection of residual plots and model validation with each above model revealed that the model assumptions were satisfied in all the cases.

Model comparison (bbmle package, Bolker, 2009) was done for the series of candidate models (1-factor models; 2-factor models; and between the best fitting 1-factor and 2-factor models), ranking them in order of plausibility based on their lowest AIC scores and highest Akaike's weights (ω), i.e. the likelihood that the candidate model is the best approximation, relative to all other models in the set of models under consideration. Models considered to be statistically plausible were selected based on ΔAIC (the difference in AIC values between a candidate model and the best performing model with the lowest AIC value) and were defined as those with $\Delta AIC \leq 2$ (i.e. not statistically different from the best performing model with $\Delta AIC = 0$) (Thomas, 2017).

3. Results:

3.1. *Cockle speciation and identification of haplosporidian species:*

The cockle speciation PCR confirmed that all the individuals from all sample sites were *Cerastoderma edule*. Overall, 27.7% (n = 277) of all cockles screened (n = 998) by PCR were positive for haplosporidian species throughout the year (Table 4).

Direct sequencing was carried out on representative PCR products amplified using generic haplosporidian HAP-F1 and HAP-R3 primers (Renault et al., 2000; Molloy et al., 2012) to identify the species present in the samples. The sequencing revealed two *Minchinia* species: *Minchinia tapetis* and *Minchinia mercenariae*-like, as discussed below.

Two sequenced individuals from Ringaskiddy (n = 1) and Cuskinny (n = 1), with a query length of approximately 300bp, showed 96-99% query coverage and 99.7-100% percent identity to *Minchinia tapetis* deposited by Hartikainen et al. (2014) (KF208603.1), and 96-99% query coverage and 98.7-99.1% percent identity to *Minchinia tapetis* deposited by Reece et al. (2004) (AY449710.1). The tested sequences showed less homology with *Minchinia mercenariae*-like sequences deposited in GenBank: < 92.3% (KY522823.1), < 92.6% (FJ518816.1).

Six sequenced individuals from Ringaskiddy (n = 1), Cuskinny (n = 2), Youghal Bay (n = 2) and Annagassan (n = 1), with a query length ranging between 248 and 318bp, displayed 96-100% query coverage and 96.7-98% percent identity to *Minchinia mercenariae* deposited by Ford et al. (2009) (FJ518816.1), and 96-100% query coverage and 99-100% percent identity to *Minchinia mercenariae*-like deposited by Ramilo et al. (2018) (KY522823.1). The latter matches exactly with the sequences found by Lynch et al. (2020). Only in one sequence, the query coverage dropped to 75%, with a query length of 393bp and a percent identity 97.3% to *Minchinia mercenariae* deposited by Ford et al. (2009), and 99.3% to *Minchinia mercenariae*-like deposited by Ramilo et al. (2018). Moreover, the tested sequences showed less homology with *Minchinia tapetis* sequences deposited in GenBank: < 91.8% (AY449710.1), < 92.5% (KF208603.1). However, it was not possible to generate forward and reverse sequences that were long enough to identify species in the rest of the sequenced samples (n = 10).

Based on the obtained sequences, two pairs of primers were developed to detect the presence of each species, since all the infected individuals could not be sequenced due to the high number of samples that were positive for haplosporidians using the generic PCR (n =

277). As validation, 24 samples were sequenced to confirm the suitability of both pairs of newly designed primers. Using MER-For/MER-Rev primers (Table 3), 12 samples were sequenced - Ringaskiddy (n = 2), Cuskinny (n = 2), Youghal (n = 2), Dungarvan (n = 1), Annagassan (n = 3) and Cooley (n = 2) -, with a query length of approximately 170bp, showing 87-91% query coverage and 96.1-99.3% percent identity to *Minchinia mercenariae* deposited by Ford et al. (2009) (FJ518816.1), and 87-91% query coverage and 96.7-100% percent identity to *Minchinia mercenariae*-like deposited by Ramilo et al. (2018) (KY522823.1). The latter matches exactly with the sequences found by Lynch et al. (2020). Furthermore, the obtained sequences showed less homology with *Minchinia tapetis* sequences deposited in GenBank: < 92.9% (AY449710.1), < 92.9% (KF208603.1). Based on the percent identity, the short length of the sequences and the low percentage of query coverage in some sequences, the species found in our samples was referred as *Minchinia mercenariae*-like, although the fragments amplified were comprised in the sequence deposited by Ford et al. (2009).

Likewise, using TAP-For/TAP-Rev primers (Table 3), 12 samples were sequenced - Ringaskiddy (n = 5), Cuskinny (n = 1), Dungarvan (n = 2), Annagassan (n = 3) and Cooley (n = 1) -, however, in the samples from Annagassan (n = 3) and in one sample from Dungarvan no significant similarity was found. The rest of samples, with a query length of approximately 165bp, showed 86-90% query coverage and 98.7-99.3% percent identity to *Minchinia tapetis* deposited by Hartikainen et al. (2014) (KF208603.1), and 86-90% query coverage and 97.9-98.7% percent identity to *Minchinia tapetis* deposited by Reece et al. (2004) (AY449710.1). Only in one sequenced sample, the percent identity dropped to 86.4% to *Minchinia tapetis* deposited by Hartikainen et al. (2014) and 85.6% to *Minchinia tapetis* deposited by Reece et al. (2004), with 80% query coverage and a query length of 165bp, without more matches present in GenBank. In general, the obtained sequences showed less homology with *Minchinia mercenariae*-like sequences deposited in GenBank: < 92.4% (FJ518816.1), < 93.1% (KY522823.1).

Based on this screening (n = 277), *Minchinia mercenariae*-like was present in 41 individuals (14.8%), and *Minchinia tapetis* was present in 82 individuals (29.6%), while 17 individuals (6.1%) displayed coinfection of both *Minchinia* spp. (Table 4). Nevertheless, 171 (61.7%) of the tested samples that were positive in the generic haplosporidian PCR did not amplify using the more specific PCR. Five of those samples were commercially sequenced in addition to the samples previously sequenced, however, no significant similarity was found in

GenBank. Therefore, the 277 positive PCR cases were defined as *Haplosporidia* spp. for statistical and analysis purposes.

Table 4: Number of PCR positive cases (positive individuals/screened individuals) for the screening of *Haplosporidia* spp. (HAP-F1/R3); *Minchinia tapetis* (TAP-For/Rev) and *Minchinia mercenariae*-like (MER-For/Rev); and the number of coinfected individuals (positive individuals/screened individuals) at each sample site by season.

Sample site	Pathogen identification	Spring 2018	Summer 2018	Autumn 2018	Winter 2018/19	Spring 2019	Totals
Cork Harbour - Ringaskiddy	*Haplosporidia* spp.	9/42(21.4%)	5/12(41.7%)	17/31(54.8%)*	0/15(0%)	3/30(10%)	**34/130**
	Minchinia tapetis	5/9(55.5%)	3/5(60%)*	8/17(47.1%)	0/0(0%)	1/3(33.3%)	**17/34**
	Minchinia mercenariae-like	9/9(100%)*	0/5(0%)	2/17(11.8%)	0/0(0%)	1/3(33.3%)	**12/34**
	Minchinia spp. coinfection	5/9(55.5%)*	0/5(0%)	1/17(5.9%)	0/0(0%)	1/3(33.3%)	**7/34**
Cork Harbour - Cuskinny	*Haplosporidia* spp.	18/85(21.2%)	1/27(3.7%)	8/36(22.2%)	8/23(34.8%)	14/30(46.7%)*	**49/201**
	Minchinia tapetis	4/18(22.2%)	0/1(0%)	3/8(37.5%)*	1/8(12.5%)	0/14(0%)	**8/49**
	Minchinia mercenariae-like	5/18(27.8%)	0/1(0%)	3/8(37.5%)*	0/8(0%)	5/14(35.7%)	**13/49**
	Minchinia spp. coinfection	2/18(11.1%)	0/1(0%)	2/8(25%)*	0/8(0%)	0/14(0%)	**4/49**
Youghal Bay	*Haplosporidia* spp.	-	1/11(9.1%)	2/16(12.5%)	17/30(56.7%)*	1/12(8.33%)	**21/69**
	Minchinia tapetis	-	0/1(0%)	0/2(0%)	0/17(0%)	0/1(0%)	**0/21**
	Minchinia mercenariae-like	-	0/1(0%)	2/2(100%)*	1/17(5.9%)	0/1(0%)	**3/21**
	Minchinia spp. coinfection	-	0/1(0%)	0/2(0%)	0/17(0%)	0/1(0%)	**0/21**
Dungarvan Harbour	*Haplosporidia* spp.	-	20/51(39.2%)	6/58(10.3%)	9/30(30%)	12/30(40%)*	**47/169**
	Minchinia tapetis	-	1/20(5%)*	0/6(0%)	0/9(0%)	0/12(0%)	**1/47**
	Minchinia mercenariae-like	-	0/20(0%)	0/6(0%)	0/9(0%)	3/12(25%)*	**3/47**
	Minchinia spp. coinfection	-	0/20(0%)	0/6(0%)	0/9(0%)	0/12(0%)	**0/47**
Dundalk Bay- Annagassan	*Haplosporidia* spp.	-	43/99(43.4%)	5/61(8.2%)	17/30(56.7%)	22/30(73.3%)*	**87/220**
	Minchinia tapetis	-	29/43(67.4%)	5/5(100%)*	0/17(0%)	0/22(0%)	**34/87**
	Minchinia mercenariae-like	-	3/43(6.9%)*	0/5(0%)	1/17(5.9%)	1/22(4.5%)	**5/87**
	Minchinia spp. coinfection	-	2/43(4.6%)*	0/5(0%)	0/17(0%)	0/22(0%)	**2/87**
Dundalk Bay- Cooley	*Haplosporidia* spp.	-	16/89(17.9%)	12/60(20%)	10/30(33.3%)*	1/30(3.3%)	**39/209**
	Minchinia tapetis	-	13/16(81.2%)*	9/12(75%)	0/10(0%)	0/1(0%)	**22/39**
	Minchinia mercenariae-like	-	4/16(25%)	0/12(0%)	0/10(0%)	1/1(100%)*	**5/39**
	Minchinia spp. coinfection	-	4/16(25%)*	0/12(0%)	0/10(0%)	0/1(0%)	**4/39**

(-) No samples

(*) The highest parasite presence (%) for each site and season

The histological screening was used as a validation method of the results from the molecular analysis. Haplosporidian infection was observed in histological sections of 67 cockles out of a subsample of 70 samples that were positive for PCR. Mostly haplosporidian parasites were observed as multinucleate plasmodia, multiple haplosporidia-like sporonts and developing spores (Figure 2; Lynch et al., 2020), and as uninucleate cells stage in lower numbers. The haplosporidia-like sporonts were predominantly visualised in the infected individuals from Ringaskiddy (Cork Harbour) throughout the year. The multinucleate plasmodia and haplosporidia-like sporonts were mainly observed throughout the connective tissue of gills and digestive gland, while the developing spores were principally found in the mantle of infected animals. Thirty histological sections screened out of the seventy were positive cases for generic haplosporidian PCR but negative for specific *Minchinia* PCRs. No obvious differences in host pathology (haplosporidian morphology, etc.) was observed between those individuals and the individuals that were positive for *Minchinia*-specific PCRs, although the strongest infected individuals observed (n = 9) were among the ones positive for *Minchinia*-specific PCRs. Additionally, 10 histological sections of samples that were negative for PCRs were screened and no evident signal of infection was found on those.

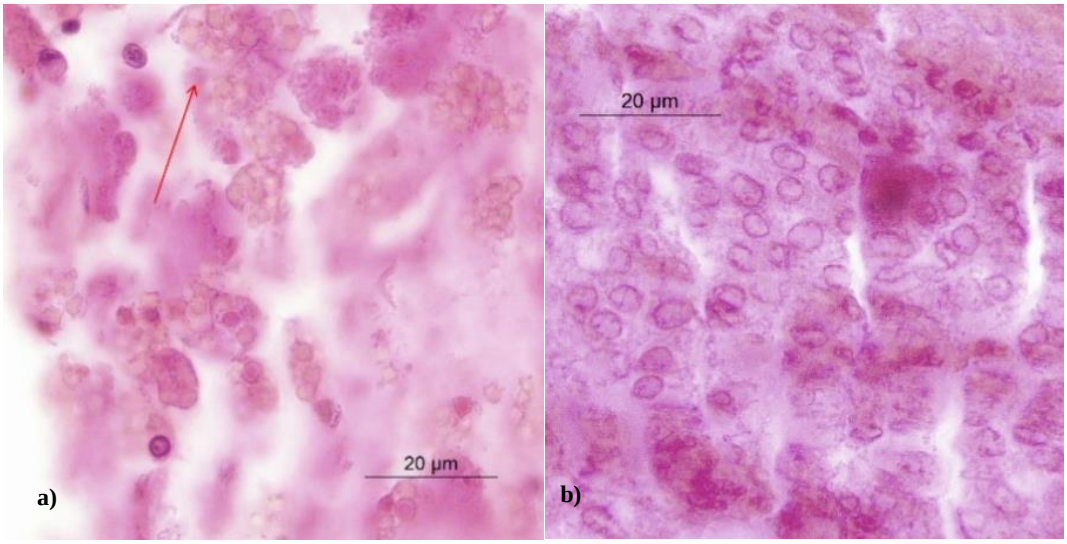


Figure 2: a) Multiple haplosporidia-like sporonts in the connective tissues of *Cerastoderma edule*; and b) a large number of haplosporidian spores in the mantle tissue of *C. edule.*

3.2. *Spatial variability in the haplosporidian presence:*

Haplosporidians were present at all the sample sites, however, differences were observed between sites. Annagassan had the highest mean presence (mean ± SD; 36.5 ±

34.8%), followed by Cuskinny (31.9 ± 24.3%), Dungarvan (29.7 ± 13.9%), Ringaskiddy (28.3 ± 21.0%), Youghal (21.7 ± 23.6%) and Cooley with the lowest mean occurrence (17.3 ± 17.1%) (Figure 3). Based on the comparison between models, the binomial GLMM considering the fixed effects of spatial and seasonal variability (Full_Model, AIC = 1154.2) had a better fit than the binomial GLMM with only the seasonal variability included as a fixed effect (Sites_Model, AIC = 1157.2), showing a significant difference between models ($p(Chisq)<0.05$) and, consequently, indicating there was a significant spatial variability on the haplosporidian distribution throughout the year.

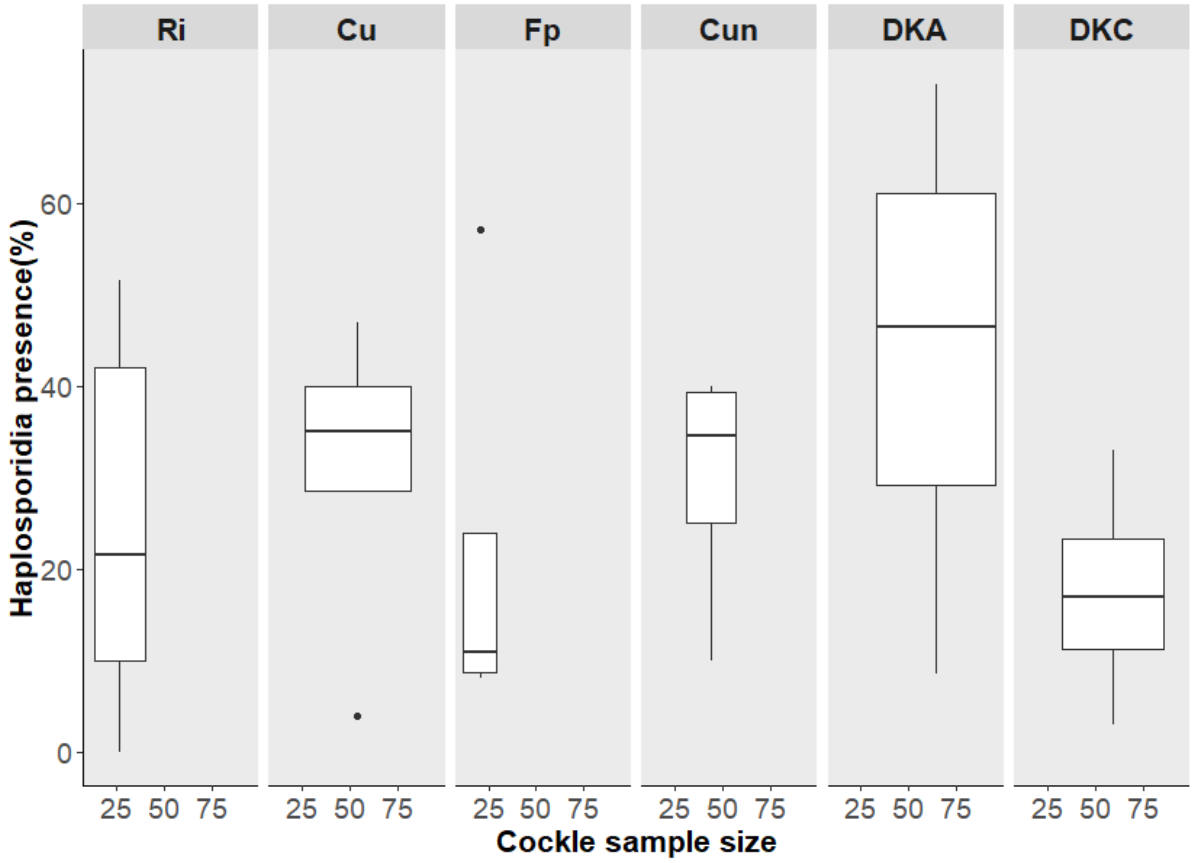


Figure 3: Haplosporidian distribution in cockles collected at each sample sites (Ri: Ringaskiddy and Cu: Cuskinny, nested sites in Cork Harbour; Fp: Youghal Bay; Cun: Dungarvan Harbour; DKA: Annagassan and DKC: Cooley, nested sites in Dundalk Bay), combining the data from all the seasons. The boxplot compactly displays the distribution of the response variable - Haplosporidia presence (%) -. The line in the box represents the median. The lower and upper hinges correspond to the 25th and 75th percentiles. The upper and lower whiskers extend to the largest and smallest values, respectively, no further than 1.5 * IQR (inter-quartile range, i.e. distance between the first and third quartiles) from the hinge.

3.3. *Seasonal variability in the haplosporidian presence:*

Although haplosporidians infecting *Cerastoderma edule* were found throughout the year, seasonality in haplosporidian infections was observed, with the highest mean presence in winter 2018/19 (mean ± SD; 35.3 ± 21.1%), followed by spring 2019 (30.2 ± 27.7%), autumn 2018 (26.3 ± 23.8%), spring 2018 (25.0 ± 18.4%), with summer 2018 (24,2 ± 25.8%) having the lowest mean presence (Figure 4). The binomial GLMM considering the fixed effects of spatial and seasonal variability (Full_Model, AIC = 1154.2) was compared to the binomial GLMM with only the spatial variability included as a fixed effect (Seasons_Model, AIC = 1169.8), showing a significant difference between models (p(Chisq)<0.001), i.e. the seasonality had a significant effect on shaping the haplosporidian distribution through the sites.

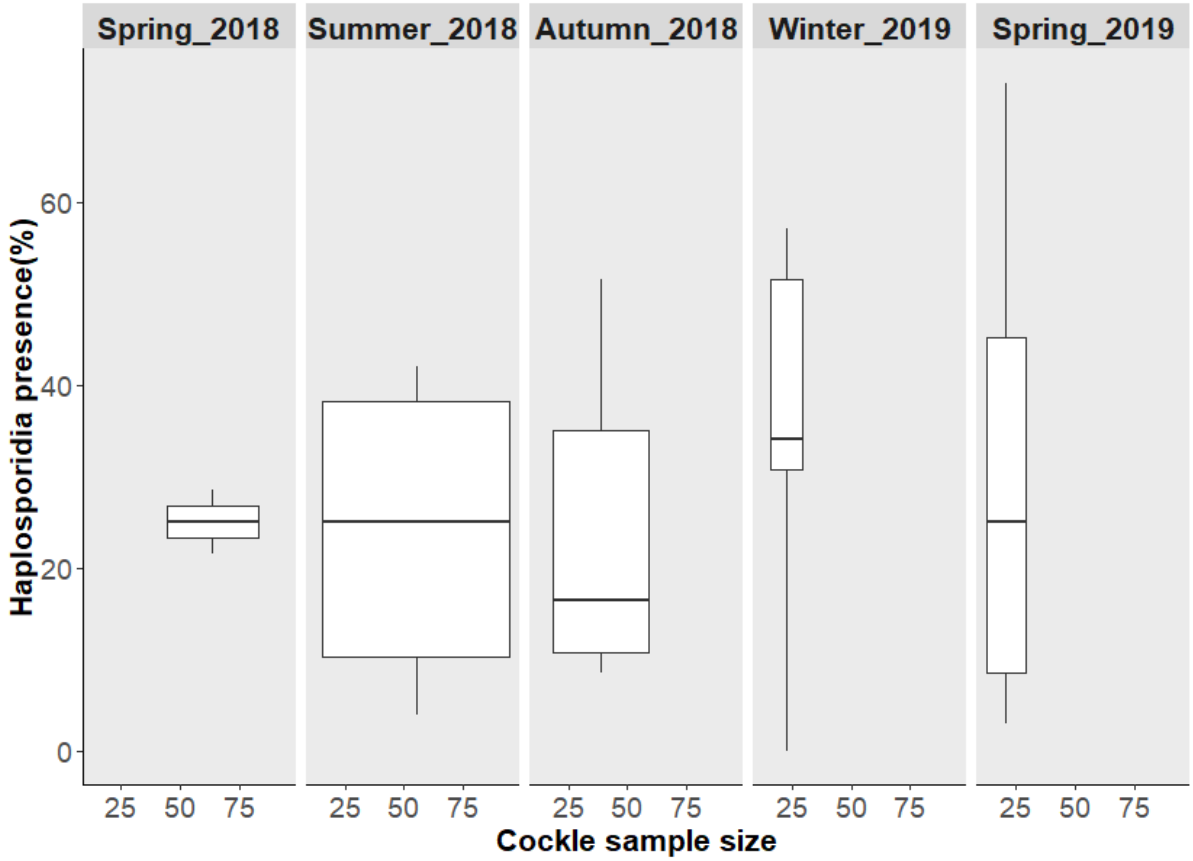


Figure 4: Haplosporidian distribution in cockles collected from different seasons (spring 2018, summer 2018, autumn 2018, winter 2018/19, spring 2019), combining the data from all the sample sites. The boxplot compactly displays the distribution of the response variable - Haplosporidia presence (%) -. The line in the box represents the median. The lower and upper hinges correspond to the 25th and 75th percentiles. The upper and lower whiskers extend to the largest and smallest values, respectively, no further than 1.5 * IQR (inter-quartile range, i.e. distance between the first and third quartiles) from the hinge.

3.4. *Effects of abiotic and biotic factors on haplosporidian infection:*

Dundalk Bay displayed higher oxygen levels, lower water temperatures and lower salinity values than the other sites, which exhibited similar values of temperature and salinity, while the oxygen levels were, on average, the lowest at both beds of Cork Harbour (Table 5). On average, the largest cockles were found at Annagassan, Cooley and Cuskinny, where the oldest cockles were found (Table 5). The smallest cockles were recorded at Dungarvan Harbour corresponding with the youngest cockles sampled (Table 5).

There was a clear seasonal variability of temperature and dissolved oxygen in water, with the lowest mean water temperature (8.5 ± 1.1°C) and a high dissolved oxygen concentration (292.0 ± 13.8 mmol/m^3) in winter, and the highest mean water temperatures (15.5 ± 1.2°C) and the lowest mean dissolved oxygen concentration (268.8 ± 12.1 mmol/m^3) in summer. While the salinity remained fairly stable during the year, with the highest value in summer (33.2 ± 1.5 PSU) and the lowest in spring 2018 (31.9 ± 1.7 PSU).

Cockle height measurements also showed a seasonal tendency. It decreased progressively from spring 2018 (33.0 ± 3.3mm) to winter 2018/19, when the lowest mean height (25.3 ± 3.6mm) was recorded, and greater cockles were subsequently sampled in spring 2019 (28.4 ± 3.2mm). In turn, cockle age showed the lowest values in summer 2018 (2.5 ± 0.7 growth rings) and winter 2018/19 (2.6 ± 0.7 growth rings).

Table 5: Mean ± SD of abiotic (temperature, salinity and dissolved oxygen in water) and biotic factors (cockle height and cockle age) at each sample site.

Mean ± SD	Cork Harbour – Ringaskiddy	Cork Harbour - Cuskinny	Youghal Bay	Dungarvan Harbour	Dundalk Bay- Annagassan	Dundalk Bay- Cooley
O_2 (mmol/m^3)	276.5 ± 13.3	275.8 ± 12.9	282.9 ± 13.4	282.2 ± 15.5	290.8 ± 22.6	295.7 ± 15.6
Temperature (°C)	11.8 ± 2.6	11.9 ± 2.6	11.9 ± 2.9	11.9 ± 2.7	11.1 ± 3.7	11.0 ± 3.5
Salinity (PSU)	33.5 ± 0.8	33.6 ± 0.7	33.9 ± 0.3	34.0 ± 0.5	30.3 ± 0.8	30.5 ± 0.7
Height (mm)	28.1 ± 4.9	29.4 ± 6.8	27.4 ± 1.1	22.3 ± 1.3	29.7 ± 1.5	29.8 ± 2.3
Age (growth rings)	2.9 ± 0.7	3.7 ± 1.4	3.1 ± 0.6	1.8 ± 0.4	2.8 ± 0.7	3.1 ± 1.2

Among the 1-factor models assessing the effects of abiotic and biotic variables (Table 6), the one considering the dissolved oxygen in the water as a fixed effect was the best fitting model (with the lowest AIC = 1122.6). Consequently, dissolved oxygen was the best performing driver explaining the haplosporidian distribution in the study. Higher haplosporidian occurrence was observed at higher dissolved oxygen concentrations. Neither of

the other two environmental factors, water temperature and salinity, came out as significant factors in determining the haplosporidian distribution, having worse fits than the null model (intercept-only model, Table 6). Nevertheless, lower temperature and salinity values corresponded to higher parasite presence.

Cockle height was the second-best explanatory factor in determining parasite presence based on the 1-factor models (AIC = 1140.4; $\Delta AIC \geq 2$, i.e. statistically less plausible than the best performing model) (Table 6). Lower haplosporidian presence was observed in larger cockles. Likewise, the 1-factor model considering the cockle age was significantly different from the null model (intercept-only model, Table 6). However, the correspondent 1-factor model showed higher AIC value (AIC = 1155.8, Table 6) than the model considering cockle height, consequently, revealing a weaker inhibitory effect on the parasite distribution than cockle height.

Table 6: Overview of the 1-factor models explaining haplosporidian presence (modelled as parasite presence/absence) infecting edible cockles *Cerastoderma edule.*

Model	**Variable**	**Estimate (β) ± SE**	**AIC**	**ΔAIC**	**Akaike's weights (ω)**
Null	Intercept	-0.975 ± 0.211	1162.9	40.3	<0.001
1-Factor	**Intercept**	**-1.111 ± 0.251**	**1122.6***	**0.0**	**1**
	Dissolved O_2 (mmol/m^3)	**0.700 ± 0.136**			
1-Factor	Intercept	-1.010 ± 0.221	1140.4*	17.8	<0.001
	Cockle height (mm)	-0.420 ± 0.086			
1-Factor	Intercept	-0.983 ± 0.208	1155.8*	33.1	<0.001
	Cockle age (growth rings)	-0.254 ± 0.086			
1-Factor	Intercept	-0.960 ± 0.231	1164.2	41.6	<0.001
	Temperature (°C)	0.116 ± 0.148			
1-Factor	Intercept	-0.975 ± 0.211	1164.9	42.3	<0.001
	Salinity (PSU)	-0.002 ± 0.128			

Best model (in bold) was selected based on its lowest AIC scores and highest Akaike's weights (ω).
(*) Significantly different from Null Model (intercept-only).

All the 2-factor models - with the top performing driver (oxygen) plus each of the other explanatory variables in turn - showed a significantly better goodness of fit than the null model (intercept-only model, Table 7). However, the model with the best goodness of fit was the one considering cockle height as a fixed factor (with the lowest AIC = 1099.9). Therefore, cockle height was highlighted as the explanatory biotic factor better determining parasite presence along with the oxygen. Lastly, the 2-factor models with water temperature, salinity and cockle

age as fixed factors disclosed weaker effects on haplosporidian presence, being the one considering the temperature the second-best performing model (AIC = 1104.4, ΔAIC ≥ 2, i.e. statistically less plausible than the best performing model) (Table 7).

Table 7: Overview of the 2-factor models explaining haplosporidian presence (modelled as parasite presence/absence) infecting edible cockles *Cerastoderma edule.*

Model	**Variable**	**Estimate (β) ± SE**	**AIC**	**ΔAIC**	**Akaike's weights (ω)**
Null_Oxygen	Intercept	-1.111 ± 0.251	1122.6	22.7	<0.001
	Dissolved O_2 (mmol/m^3)	0.700 ± 0.136			
2-Factor	**Intercept**	**-1.146 ± 0.250**	**1099.9***	**0.0**	**0.901**
	Dissolved O_2 (mmol/m^3)	**0.695 ± 0.131**			
	Cockle height (mm)	**-0.431 ± 0.087**			
2-Factor	Intercept	-1.097 ± 0.342	1104.4*	4.5	0.097
	Dissolved O_2 (mmol/m^3)	1.148 ± 0.214			
	Temperature (°C)	0.752 ± 0.202			
2-Factor	Intercept	-1.113 ± 0.234	1113.8*	13.8	<0.001
	Dissolved O_2 (mmol/m^3)	0.689 ± 0.141			
	Cockle age (growth rings)	-0.287 ± 0.089			
2-Factor	Intercept	-1.232 ± 0.291	1115.9*	15.9	<0.001
	Dissolved O_2 (mmol/m^3)	0.882 ± 0.164			
	Salinity (PSU)	0.541 ± 0.223			

Best model (in bold) was selected based on its lowest AIC scores and highest Akaike's weights (ω).
(*) Significantly different from Null Model (intercept-only).

A comparison between AIC values of the best fitting 1-factor (Table 6) and 2-factor (Table 7) models revealed that, overall, the 2-factor model with oxygen and cockle height as fixed factors was the most plausible explanatory model defining the haplosporidian species distribution in the study. The rest of the models were considered statistically different from the former one (ΔAIC ≥ 2) and, consequently, less plausible.

4. Discussion

For the first time, a comprehensive study assessing the temporal and spatial distribution of haplosporidian species in commercial and non-commercial *Cerastoderma edule* populations at a new geographic range in Ireland was carried out. These findings further support that the *Minchinia mercenariae*-like haplosporidian and *Minchinia tapetis* infect *C. edule* of all sizes and age groups in Europe (Carballal et al., 2001; Villalba et al., 2001; Carballal et al., 2003; Engelsma et al., 2011; Longshaw and Malham, 2013; Ramilo et al., 2018). Similar to other haplosporidian species (Ford and Haskin, 1982; Burreson et al., 2004; Engelsma et al., 2010; Lynch et al., 2014), the *Minchinia* spp. detected in the study were present year-round at all sites, except for *M. tapetis* in Youghal. Coinfection of both *Minchinia* species was only observed in Cork Harbour and Dundalk Bay, where *Minchinia* spp. showed a higher presence compared to Youghal Bay and Dungarvan Harbour (Celtic Sea).

Certain samples that were deemed positive in the generic haplosporidian PCR did not amplify a product in the species-specific PCRs. The discrepancy between the results from the generic haplosporidian and *Minchinia*-specific screenings could indicate the presence of other haplosporidian species in the samples. The absence of amplicons in the *Minchinia*-specific screening could also be due to the low intensity of infection observed in the cockles by histology. A low quantity of pathogen DNA present in the mixed (host and pathogen DNA if present) samples would reduce the probability of detecting the target *Minchinia* DNA during PCR. It could also be attributed to gradual DNA degradation in the gill tissue samples due to the freeze thawing process.

Overall, more than a quarter of the *Cerastoderma edule* at the Irish sample sites were positive for haplosporidians. Spatial variability was observed between sites and even between cockle beds within the commercial fishery (Annagassan and Cooley) at Dundalk and the marine nature reserve and shipping port (Cuskinny and Ringaskiddy) within Cork Harbour.

Both cockle beds at Dundalk Bay displayed similar environmental conditions and cockles there presented similar biotic characteristics, but while Annagassan, exhibited, on average, the highest haplosporidian presence, Cooley was the sample site with the lowest haplosporidian presence. Although *Cerastoderma edule* at both Dundalk beds experienced the same mean salinity year-round when the tide was in, the Cooley bed is located higher up the bay, is in close proximity to the mouth of a river, and therefore experiences more freshwater runoff when the tide is out compared to the Annagassan bed. Therefore, it was hypothesised

that salinity could have a potential effect in defining haplosporidian distribution on that bay. It has long been known that spring freshwater runoff is an important control of *Haplosporidium nelsoni* in its host the eastern oyster *Crassostrea virginica* in North America (Ford and Haskin, 1982; Burreson and Ford, 2004). Ford and Haskin (1988) highlighted that reduced prevalence of *H. nelsoni* in *C. virginica* held at a low salinity was probably due to a physiological inability of the parasite to tolerate reduced salinity rather than an enhancement of host defence mechanisms. Flannery et al. (2014) observed a lower prevalence of another haplosporidian, *Bonamia ostreae,* in the European flat oyster *Ostrea edulis* on oyster beds located close to a freshwater source at two different sample sites. These findings would suggest that the haplosporidian species are limited by reduced salinity, despite this was not highlighted as a relevant factor in our models. Older and larger sized *Cerastoderma edule* were sampled at the Cooley bed, which would indicate that low haplosporidian detection in Cooley was not due to cockle mortality induced by the freshwater runoff, as adult cockles are able to close their valves to reduce exposure to conditions of unfavourable salinity that extend days to weeks (Coen and Bishop, 2015), but more than likely an intolerance of the haplosporidians to low salinity. Continuous sampling at a time scale, rather than punctual sampling, would be necessary, to definitively confirm the role of salinity on parasite infection and transmission at a population level.

At the two cockle beds within Cork Harbour, *Cerastoderma edule* at Cuskinny Marsh Nature Reserve had a significantly higher overall haplosporidian occurrence compared to *C. edule* at Ringaskiddy, contrary to what might be expected given the proximity of Ringaskiddy to an industrial and shipping area. It is recognised that coastal development and anthropogenic disturbances may unbalance the interplay between infectious disease agents and their hosts in marine ecosystems (Rowley et al. 2014; Coen and Bishop, 2015). The *Cerastoderma edule* sample size at Cuskinny was greater than Ringaskiddy possibly indicating a lower *C. edule* density or that a mortality event may have occurred at that site. While the environmental conditions were similar at both beds of Cork Harbour, the cockle size at Cuskinny was greater than at Ringaskiddy, which is contrary to the fact that cockle height was highlighted as a significant explanatory factor inhibiting the haplosporidian infection. Therefore, although cockle age was not stressed as a key factor in our models, it has been inferred that the oldest cockles sampled at Cuskinny were exposed for a longer period of time to the haplosporidians, thus increasing their likelihood of becoming infected and having a higher occurrence of this

pathogen. It is well known that older hosts have more time than younger hosts to accumulate disease-causing pathogens (Lafferty and Kuris, 2009; Breitburg et al., 2015).

Seasonal variability was also confirmed, the highest peak of parasite presence in winter was related to the higher dissolved oxygen concentration in the water (the best performing driver in our models); conversely, in summer with the lowest mean dissolved oxygen concentration, the cockles showed the lowest mean parasite presence. It would be expected that a lower dissolved oxygen environment would be more stressful to *Cerastoderma edule* and would make them more susceptible to pathogen infections. Emerging diseases have been previously linked to climate change and other anthropogenic stressors, extreme temperatures and salinities, and low dissolved oxygen (e.g., Harvell et al., 1999; Harvell et al., 2002; Lafferty et al., 2004). Prolonged exposure to hypoxia has been shown to increase pathogen-related mortality and impair immune responses in a number of marine and estuarine invertebrates (Robohm et al., 2005; Patterson et al., 2014; Breitburg et al., 2015). As it has been observed in previous studies, when the temperature rises, oxygen levels decrease and metabolic rates increase, suggesting that warming could lead to respiratory stress, thus increasing organism's susceptibility to disease (Lafferty et al., 2004; Mackenzie et al., 2014). It was suggested that a possible explanation of this positive relation between dissolved oxygen and haplosporidian infection in the study may be associated with an increased water circulation/turbidity, therefore increasing the probability of host contact with the pathogens. Nevertheless, further research must be done in that sense to reach a clear conclusion.

Even if the seawater temperature was not defined in the 1-factor models as an important factor determinant of the parasite distribution, a higher parasite presence was observed in the cooler winter months. Additionally, a higher overall haplosporidian presence was detected in the cooler spring 2019 compared to spring 2018 in this study. Contrary to our results, simulations using progressive cooling or warming conditions made by Burreson and Ford (2004), under high salinity conditions, indicated that low temperature conditions caused a dramatic reduction in the prevalence of *Haplosporidium nelsoni* in *Crassostrea virginica*. Conversely, a higher prevalence of *Bonamia ostreae* in *Ostrea edulis* was associated with cooler spring temperatures over a thirty-year study period in Ireland (Engelsma et al. 2010; Lynch et al., 2014). These findings would suggest that a thermal tolerance range exists for haplosporidian species.

In addition, it was observed that the height of cockles sampled, the second-best explanatory factor in determining parasite presence, also showed a seasonal tendency, with the

lowest mean height and the highest mean parasite presence recorded in winter, and greater cockles subsequently sampled in spring 2019, corresponding with a decrease in parasite occurrence. As it has been previously reported, the host size is a relevant factor for the disease infection and transmission, it is commonly accepted that larger hosts, which typically eat more than their smaller counterparts, have a greater probability of consuming parasites while feeding (Mouritsen et al., 2003; Breitburg et al., 2015). The greater reproductive output of larger/older individuals may, during times of reproduction, cause a reduction in host condition and reduction of energy available for immunological defence, thereby rendering them more susceptible to infections (Taskinen and Saarinen, 1999). Nevertheless, the susceptibility of bivalves to viral and bacterial infections is generally greater for smaller younger individuals than for larger adults (Renault and Novoa, 2004), since larger/older bivalves have had more time available to develop defence mechanisms compared to their smaller/younger cohorts (Coen and Bishop 2015).

Findings from this study provide a better knowledge on novel haplosporidian species and a better understanding of some of the biotic and abiotic factors influencing haplosporidian species, particularly, *Minchinia* spp. in commercially fished and wild *Cerastoderma edule.* Both spatial and temporal variation indicated that site influence and seasonal environmental parameters play a role in haplosporidian species distribution and possibly *Cerastoderma edule* tolerance to the haplosporidians. Spatial variability occurred within a bay and harbour in this study, thus highlighting that localised site influences can have an impact. In our study, haplosporidians appeared to have a tolerance range for the environmental variables assessed (dissolved oxygen, temperature and salinity), which is typical of a diverse range of haplosporidian parasites. All age groups and size classes of *Cerastoderma edule* were associated with haplosporidians, although smaller and older cockles seemed to be more vulnerable to the infection.

These results will contribute to an improved understanding of such an important emergent pathogen in molluscs as Haplosporidia, its diversity, occurrence, seasonality, host and geographic distribution and the potential drivers/inhibitors of emerging infectious diseases associated with haplosporidian species in cultured and non-cultured *Cerastoderma edule* populations.

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