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Cockle Health, Disease Connectivity and Trophic Interaction Dynamics

Thesis presented by

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Abstract

Parasites and pathogens are an essential part of the community structure, and their transmission and development are affected not only by their host's immune system and host population density, but also by multiple biological and environmental drivers that impact on the host-parasite dynamics. There are many gaps in the understanding of these processes and how they influence one another. Given their ecological and economic importance and their exposure to fluctuating and stressful coastal environments, common cockles *Cerastoderma edule* are ideal model organisms to investigate these dynamics within a natural framework. Therefore, this thesis is intended to present *C. edule* as a model pathosystem to study marine epizootics and their increased risk of occurrence due to the current changing environment. This thesis develops the understanding of the biological and environmental factors that influence the distribution and dynamics of the host-parasite interplay. In this study, single and multiple parasite and pathogen infections plus over a range of study areas were utilised to better understand these dynamics. The initial part of the study describes a previously undocumented geographic range on the northeast and south coasts of Ireland of two haplosporidian species, *Minchinia tapetis* and *Minchinia mercenariae*-like, that have been recently characterised in *C. edule*. In order to understand the *Minchinia*:cockle disease dynamics, species-specific primers were developed and these parasites were detected by PCR and confirmed by sequencing. The histology, in turn, confirmed the presence of spore-like stages in the connective tissues of *C. edule.* A range of factors including host condition and environmental drivers affected both spatial and temporal patterns of these haplosporidian infections in *C. edule*. Based on statistical modelling, high dissolved oxygen in seawater and small cockle size were the main drivers of the haplosporidian prevalence in the study. The detailed examination of a wider microbial community within *C. edule* by PCR and histology revealed that single infections with Haplosporidia or *Vibrio* were more common than coinfected individuals, as expected by random chance. During this second study, no microsporidian species nor ostreid herpesvirus-1 microVar (OsHV-1 µVar) or variants were detected. Based on statistical modelling, coinfected individuals with Haplosporidia and *Vibrio* occurred because the same risk factors (increased seawater temperature, reduced salinity, and poor host condition) promoted their presence and increased the probability of infection by both pathogen groups. Coinfection with both

Minchinia species was overrepresented in our samples and a positive association between *M. tapetis* and *M. mercenariae*-like was statistically confirmed. Findings emphasise the need for a more holistic approach in pathological studies, considering the diverse pathogen community within the cockle and their relationship with the environment. The transmission of the microbial community within *C. edule* to shorebird populations via consumption of cockles and the existing connectivity with the environment (sediment) were also assessed by molecular techniques (PCR and Sanger sequencing) during a third study. Identical strains of *Vibrio splendidus* were identified in *C. edule* and, for the first time, in shorebird faecal samples. The consumption of *C. edule*, thus, may be a source of this *Vibrio* species in shorebirds. Our findings add evidence to the significant role migratory birds may have as carriers of infectious agents, enabling their transport and dispersal. The findings also support the role of the sediment as an environmental reservoir for *Vibrio*, which may have an impact on the infaunal community within the sediment responsible for different ecosystem services. Ultimately, in a study that spanned the east and west coast of Ireland and the Welsh coast, patterns of occurrence of *Minchinia* and *Vibrio* spp. in *C. edule* were associated with environmental stressors (high concentration of nitrates and warm temperatures), which may have affected host susceptibility and increased pathogen prevalence. These findings highlight the impact of site-specific environmental stress and habitat degradation on pathogen emergence, particularly in coastal marine environments. In conclusion, this thesis develops a greater understanding of *C. edule* as a pathosystem and its potential for the early detection of emergent infectious agents, which will have important implications for the sustainability of wild and cultured bivalve populations and the functioning of coastal ecosystems in a changing environmental context.

Outline

This is a Publication-Based Doctor of Philosophy (PhD) Thesis. I am the first author and key contributor to this work. Individual author contributions are specified for each manuscript. The thesis contains seven chapters. *Chapter 1* is a general introduction to host-parasite ecology. The model species used in this thesis, the common cockle *Cerastoderma edule*, is presented and the general aims of the study are listed. *Chapter 2* consists of a literature review of host-parasite interplay in *C. edule* viewed as a pathosystem. Published literature is discussed with a focus on the reciprocal interaction between the host, the parasite and the environment, its impact on ecosystem stability and the transmission routes used by parasites within a natural framework. *Chapter 2* has been prepared to be submitted as a published review. *Chapters 3 - 6* present the primary research of the thesis. Given the recent description of the haplosporidian species *Minchinia mercenariae*-like affecting *C. edule* on the Irish coast (Lynch et al., 2020), *Chapter 3* describes the investigation of emergent haplosporidian species in *C. edule* at study sites along the Irish coastline in the Irish and Celtic Seas, with a focus on the pathogen distribution and seasonal effect and the interplay of abiotic and biotic drivers/inhibitors of infection. This study has been published in the *Journal of Invertebrate Pathology* (doi: 10.1016/j.jip.2020.107425). In *Chapter 4* we broaden the microbial examination to other pathogen groups, such as bacteria, virus, and Microsporidia, that can have a detrimental effect on *C. edule* health, emphasising that pathogen assemblages can exist within the host and the effect of biotic and environmental drivers on the risk of coinfection. This chapter has been published in *Parasitology* (doi: 10.1017/S0031182021001396). *Chapter 5* investigates if the microbial community within *C. edule* can be transmitted to shorebird populations via cockle consumption and the existing connectivity with the environment (sediment). This manuscript has been published in *Scientific Reports* (doi: 10.1038/s41598-021-01610-x). *Chapter 6* provides insight into the spatial patterns of the microbial pathogens present in *C. edule* on a wider geographic range across Ireland and Wales. *Chapter 6* emphasises that complex patterns of interactions are observed between the host-pathogen system and several localised habitat conditions (e.g., temperature, nutrients, host density, anthropogenic impacts), which

influence spatial pathogen distribution in exploited and non-exploited *C. edule* populations. Lastly, *Chapter 7* discusses key conclusions and their implications in relation to current scientific understanding and the potential for new research not only on *C. edule* biology and ecology but on all marine bivalve species.

CHAPTER 1

General Introduction

Introduction

Parasites and pathogens are ubiquitous and represent a substantial portion of the species diversity and biomass in food webs and ecosystems (Hudson et al., 2006; Poulin, 2010; Selakovic et al., 2014). Parasites and pathogens live at the expense of the host and divert host energy from growth and reproduction into disease resistance, impacting host population dynamics (Selakovic et al., 2014). Besides, their effects may also trigger parasite-mediated trophic cascades (Hatcher et al., 2012), shaping food webs and modifying their structure and stability in different ways (Hudson et al., 2006; Lafferty et al., 2008). Parasites and pathogens, in turn, depend on the ecological networks in which they live, with the disease dynamics affected by food web structure (Lafferty et al., 2008). Processes at different scales of ecological organization from the within-host level (e.g., interactions of coinfecting parasites) to the ecosystem level (e.g., the influence of environmental variables) across space and over time may have a key influence on complex natural systems (Hellard et al., 2015). Therefore, the host-parasite interplay may be viewed as a pathosystem, an ecological subsystem within an environment, which is defined by the reciprocal interaction between the host, the parasite, and the environment (Sullivan et al., 2013).

Given the position of bivalves in trophic webs as filter feeders, their key function in different ecosystem services and their role as hosts of a wide range of parasites and pathogens (Mouritsen and Poulin, 2002; Rowley et al., 2014; Carss et al., 2020), this animal group and, particularly, common cockles *Cerastoderma edule* are ideal model organisms for pathosystem studies. Cockles are ecosystem engineers, they occur intertidally and subtidally in estuaries and sandy bays, where they are exposed to fluctuating and stressful environments, which may alter the host-pathogen interplay and affect both spatial and temporal patterns and the severity of epizootics (Breitburg et al., 2015).

Characterising the diversity and distribution of microbial parasites/pathogens in hosts such as *C. edule* remains a challenge due to their patchy spatial and temporal distributions, host-restricted occurrence, and poorly known life cycles (Hartikainen et al., 2014; Lynch et al., 2020). Despite that, emergent microbial agents, such as emergent haplosporidians (obligate protozoan parasites) (Ramilo et al., 2018; Lynch

et al., 2020), continue to be reported from many different invertebrate hosts and habitats, highlighting the importance of the microbial parasites and pathogens, the study of which has been neglected comparing to macroparasites, such as trematodes.

Despite research into host-parasite interactions being primarily dominated by the study of one host–one parasite systems, animal hosts are exposed to complex parasite/pathogen assemblages that ultimately form a dynamic community within them (Johnson and Buller, 2011). Interactions among coinfecting agents can, in fact, alter host pathology, parasite transmission and virulence evolution, also influencing the spread of infections at a population level resulting in disease outbreaks, as described previously in bivalves (Lassalle et al., 2007; Arzul et al., 2012; Alfaro et al., 2019). Simultaneous infections may trigger a whole spectrum of outcomes within the hosts, both synergistic and antagonistic, i.e., the impact of one or both infectious agents may be suppressed (Fukami et al., 2010), one or both agents may be amplified resulting in parasite coexistence (Thomas et al., 1998), or one may be amplified and the other suppressed (Johnson and Hoverman, 2012). The co-occurrence of parasites/pathogens may also result simply because the same risk factors promote their presence and not because the different parasite/pathogen groups are interacting synergistically (Vaumourin et al., 2015). Given the complex microbial assemblages within *C. edule* and their intricate interactions and effects, a comprehensive and integrated approach is needed to shed light on the disease dynamics in natural systems. Not only must the synergistic impact of coinfections be considered, but also the role of ecological factors driving the infection across space and over time.

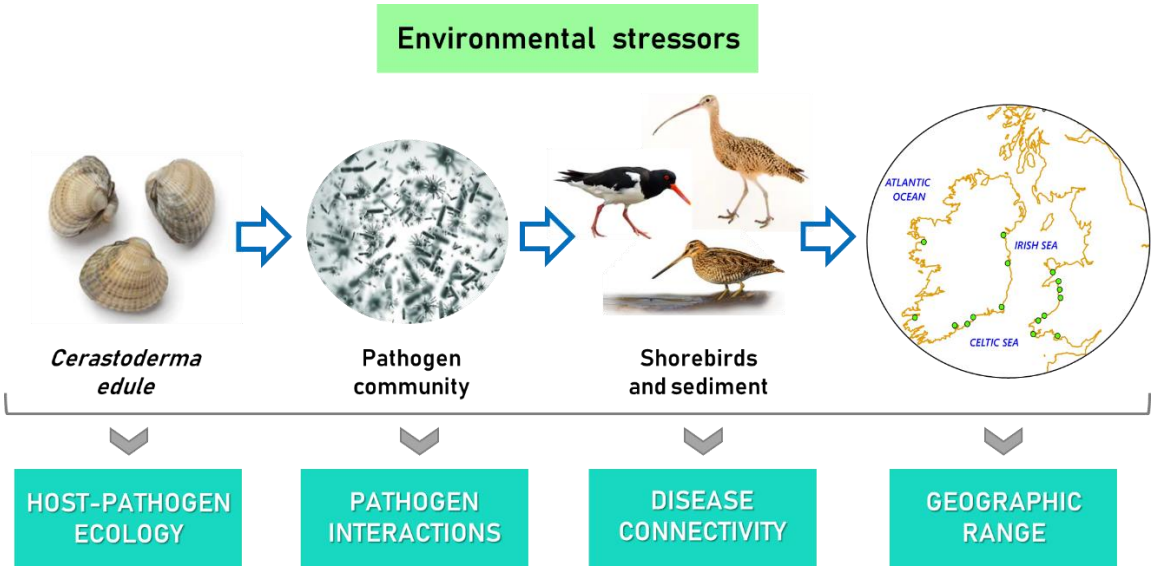
Parasites and pathogens can be trophically transmitted via predator-prey interactions (Coen and Bishop, 2015). Concomitant predation is the most common way for parasites and pathogens to become prey, which occurs when an infected host is eaten by a predator (Johnson et al., 2010). In the marine food chain, *C. edule* are an important food source and are a link between primary producers and consumers, playing a crucial role in the ecosystem (Malham et al., 2012; Carss et al., 2020). One of the main consumers feeding on up to 300 cockles per day is marine bird populations (Malham et al., 2012), which may be involved in the carriage of microbial pathogens (Hubálek, 2004). Because of the high productivity of infectious agents, their unique nutritional composition and their pathogenicity in hosts, their consumption affects food web structure, energy transfer and disease distribution (Johnson et al., 2010). An

integrative examination is essential to better understand the underlying mechanisms of disease connectivity, including the trophic transmission of microbial infections between cockles and shorebirds.

As a result of increasing anthropogenic stressors, there has been an increase in emerging infectious diseases in marine ecosystems, referring to emergent diseases as the ones that have recently increased in incidence or impact, or have recently moved into a new host population or geographic region (Lederberg et al. 1992, Smolinski et al. 2003; Lafferty et al., 2004; Newman et al., 2007). Strong links have been established between disease and climate in marine invertebrate populations (Marcogliese, 2008). There is increasing evidence that climate change will affect the ecology of infectious diseases and the physiology of bivalve species and their resistance to infection (Soon and Zheng, 2020). However, there are still many gaps in the understanding of these processes, especially regarding microbial pathogens.

It is crucial, therefore, to improve our knowledge of *C. edule* pathosystem effects on all levels of the ecological organization to achieve a better understanding of host-parasite dynamics and interactions, in particular in the current scenario of climate change that has and will have a major impact on coastal marine ecosystems.

Main aims of the study:



➢ Chapter 2 reviews the available literature concerning the host-parasite ecology in bivalves, with special attention to *Cerastoderma edule*, and the disease-mediated effects and transmissibility, considering the role of the environment on host-parasite dynamics.

➢ Chapter 3 determines the diversity, spatial patterns, and temporal variability of emergent haplosporidian species, *Minchinia tapetis* and *Minchinia mercenariae*-like, in *C. edule* populations along the Irish coastline of the Irish and Celtic Seas, assessing the abiotic and biotic drivers or inhibitors of the infection.

➢ Chapter 4 examines the diversity and prevalence of significant pathogen groups - Haplosporidia, *Vibrio* spp., ostreid herpesvirus-1 microVar (OsHV-1 µVar) and Microsporidia - as well as coinfections and associations of the different infectious agents within wild and fished *C. edule* along the Irish coastline. The effect of key biotic and environmental factors on the risk of coinfection across space and over time is assessed.

➢ Chapter 5 assesses if pathogens associated with *C. edule* - *Vibrio* spp. and Haplosporidia spp. - could be detected seasonally along the Irish coastline in the

faeces of shorebirds that feed on *C. edule* and in the physical environment (sediment) in which *C. edule* reside. Spatial and seasonal influence in the detection of the infectious agents in cockles, shorebird faecal samples as well as sediment is considered.

- Chapter 6 examines the spatial patterns of emergent pathogens – *Minchinia tapetis*, *M. mercenariae*-like and *Vibrio* spp. - associated with exploited and non-exploited *C. edule* populations at a wider geographic range along the Irish and Welsh coastlines. Some of the ecological factors that may be shaping the pathogen distribution and dispersal are determined.

- Chapter 7 highlights the implications derived from this study for the sustainable management of coastal ecosystems. The study has raised a number of important future research questions on *C. edule* and marine bivalve biology and ecology.

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CHAPTER 2

Bivalve pathosystems support a better understanding of host-parasite ecology and its impact on ecosystem stability

Bivalve pathosystems support a better understanding of host-parasite ecology and its impact on ecosystem stability

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Abstract

The emergence, spread, and persistence of marine infectious agents can have important effects on community structure and function by modifying individual and population-level traits of hosts, particularly if hosts are ecosystem engineers or habitat-forming species such as bivalves. This review, therefore, emphasises the importance of understanding the functioning of host-parasite systems within the marine environments and their impact on the ecosystem, using *Cerastoderma edule* as a model pathosystem. The pathosystem concept, considered as a subsystem within an environment and defined by the phenomenon of parasitism, has never been associated with cockles, to the best of our knowledge. However, *C. edule*, as a bioindicator species with its prominent role in different ecosystem services and widespread distribution, can improve our knowledge of host-parasite dynamics and the interaction with the current changing marine environment. Therefore, this review is intended to present *C. edule* as an ideal model pathosystem in order to study the marine epizootics and the biological and environmental factors that influence the distribution and dynamics of the host-parasite interplay within a natural framework. This work synthesises how infectious agents, as an integral part of the ecosystem, are

influenced not only by the host immune system and density but also by the interactions between non-host populations and the environment. This review highlights the importance of integrated research, with particular regard to how pathosystems influence the assembly, functioning and stability of communities and ecosystems. Further integrating these fields will have important consequences for the sustainability of wild and cultured bivalve populations and the functions of nearshore ecosystems.

Keywords: *Cerastoderma edule*, pathosystems, host-parasite ecology, parasite transmission, environmental stressors.

2.1. Introduction

The host-parasite interplay may be viewed as a pathosystem, an ecological subsystem within an environment, which is defined by the reciprocal interaction between the host, the parasite and the environment (Sullivan et al., 2013; Figure 1). The concept of pathosystems has been popular in plant pathology (e.g., Robinson et al., 1976; Zhan et al., 2002; Schroeder et al., 2019; Duffin et al., 2020), however, studies related to pathosystems and their ecological dynamics in aquatic ecosystems are scarce (Sullivan et al., 2013; Jephcott et al., 2016), due to their often unpredictable and inconspicuous nature. This review, thus, stresses the importance of understanding the functioning of pathosystems in marine environments and their impact on ecosystem balance and evolution. It is crucial to improve our knowledge of pathosystem effects on all levels of ecological organization from individuals to ecosystems to achieve a better understanding of host-parasite dynamics and interactions, in particular in the current changing environmental context that is having a major impact on the marine ecosystem.

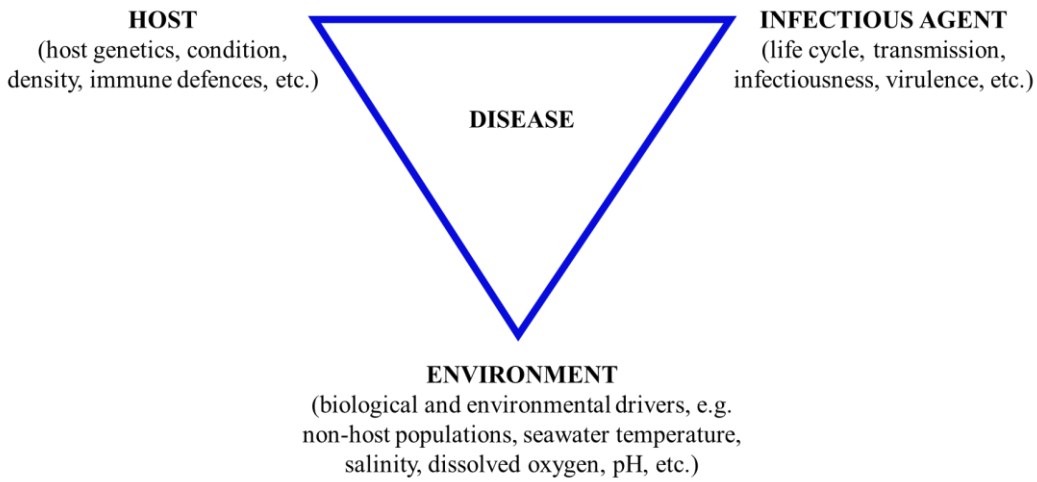

Figure 1: A generalised pathosystem conceptual model. Host, infectious agent, and the environment are placed at the corners of an epidemiological triangle, representing their reciprocal interaction. The interaction among these three components may cause disease.

Infectious agents can have large effects on community structure and function by modifying individual and population-level traits of hosts, particularly if hosts are ecosystem engineers or habitat-forming species such as bivalve molluscs (Thomas et

al., 1998). Bivalves may serve as an intermediate or definitive host for multiple parasites and pathogens (Coen and Bishop, 2015). Causation between diseases and significant mortality events is often difficult to establish in aquatic environments. Nevertheless, mortality of bivalves has been associated with diseases since the 1950s, linked particularly to protists such as *Perkinsus* (Novoa et al., 2002; Carella et al., 2020), *Marteilia* (Carrasco et al., 2015; Kerr et al., 2018), *Haplosporidium* (Burreson and Ford, 2004; Panarese et al., 2019; Carella et al., 2020) and *Bonamia* (Culloty and Mulcahy, 1996; Culloty and Mulcahy, 2007; Zannella et al., 2017). The mortality of bivalves has been also linked to viruses (Renault and Novoa, 2004; Alfaro et al., 2019; Richard et al., 2020), bacteria (Petton et al., 2015; Alfaro et al., 2019; Rojas et al., 2021), macroparasites such as trematodes (Thieltges, 2006; Morgan et al., 2012), and non-native species (Carnegie and Burreson, 2011; Costello et al., 2021), as well as to environmental triggers (Paillard et al., 2004a; Polsenaere et al., 2017; Alfaro et al., 2019).

Bivalve molluscs have proved to be useful bioindicators and model organisms, for pathological studies, due to their filter-feeding behaviour, importance in the marine food web, prominent role in different ecosystem services, widespread distribution, and ease of sampling. In that sense, marine bivalve pathosystems can serve as ideal models to highlight the activity of agents causing emerging infectious diseases in fluctuating environments, and the co-evolutionary processes that define the host-parasite relationship. Being emerging diseases defined as the ones that have recently increased in incidence or impact or have recently moved into a new host population or geographic region (Lederberg et al. 1992, Smolinski et al. 2003; Lafferty et al., 2004; Newman et al., 2007).

Among bivalves, cockles, family Cardiidae, have been widely used as target species in research, given their role in different ecosystem services, i.e., carbon storage, energy cycling, bioturbation, a food source for seabirds, etc. (Morgan et al., 2013; Carss et al., 2020) and their economic value (Rowley et al., 2014; Carss et al., 2020). Particularly, the common cockle *Cerastoderma edule* (Linnaeus, 1758) is one of the most common infaunal bivalve species in many northern European soft-sediment coastal waters, often reaching high densities, and is commercially important in some areas of its geographic range, from the Barents Sea to Senegal, Africa

(Malham et al., 2012; Longshaw and Malham, 2013; Morgan et al., 2013; Burdon et al., 2014). *C. edule* are suspension feeders and an important food source within the marine trophic web (Malham et al., 2012; Longshaw and Malham, 2013). Consequently, they are a link between primary producers (phytoplankton, phytobenthos) and consumers such as crabs, shrimp, fish, and birds, playing a crucial role in the ecosystem (Malham et al., 2012; Carss et al., 2020).

Even though *C. edule* has been used as a model system in previous studies related to parasitism (Malham et al., 2012; Longshaw and Malham, 2013; Burdon et al., 2014; Rowley et al., 2014), the pathosystem concept, to the best of our knowledge, has not been investigated in cockles. The most well-studied molluscan host-parasite systems are those that involve commercially cultivated species, such as the Pacific oyster *Crassostrea gigas*. However, wild-harvested species such as the common cockle *C. edule* have largely been neglected in the investigation of marine epizootics. Therefore, this review is intended to cover this paucity of knowledge and present *C. edule* as an ideal model pathosystem in order to study the biological and environmental factors that influence the distribution and dynamics of the host-parasite interplay within a natural framework. A better understanding of the host-parasite ecology within the natural framework rather than under controlled conditions is crucial, given the increased risk of occurrence of marine epizootics due to the current changing environment (Zgouridou et al., 2021).

2.2. Model pathosystem

Mortalities and catastrophic declines in *C. edule* populations have been related to disease, climatic events, predation, pollution, failed recruitment, and overfishing (Malham et al., 2012; Burdon et al., 2014; Magalhães et al., 2017). However, given the complex interactions between biological and ecological factors and the scarcity of data collected, it is difficult to unequivocally assign a cause/s to host deaths in mortality events (Malham et al., 2012). Nevertheless, strong relationships between the timing of significant mortality events in wild populations of bivalve molluscs and parasite loadings in surviving individuals are highly suggestive of disease-mediated population control (Coen and Bishop, 2015). Other evidence of disease control on

bivalve population dynamics is the spatial contrasts of mortality rates between infected and uninfected populations of a diverse array of bivalve species (Villalba et al., 1993; Thieltges and Reise, 2007; Ramilo et al., 2014).

C. edule may be host to multiple micro-parasite infections including protists, bacteria, and viruses (Longshaw and Malham, 2013), but *C. edule* may also be infected by a range of macroparasites, trematodes being particularly common (Fermer et al., 2011b) (Figure 2). One protistan pathogen group that *C. edule* is known to be susceptible to is the Haplosporidia, which are widespread and have been associated with some of the most serious epizootic mortalities of commercially important molluscs (Burreson and Ford, 2004; Longshaw and Malham, 2013; Carnegie et al., 2016). Three out of the four known haplosporidian genera have been detected in *C. edule*: *Minchinia* (Ramilo et al. 2018; Albuixech-Martí et al., 2020; Lynch et al., 2020), *Haplosporidium* (Azevedo et al., 2003; Engelsma et al., 2011), and *Urosporidium* which parasitises trematodes or turbellaria infecting cockles (Carballal et al., 2005); while the genus *Bonamia* is a serious pathogen of oysters (Culloty and Mulcahy, 1996; Engelsma et al., 2014). Both direct and indirect transmission has been associated with haplosporidian species, with most haplosporidian parasites being at a low prevalence of infection in host populations (Burreson and Ford, 2004; Arzul and Carnegie, 2015; de Montaudouin et al., 2021). Haplosporidia life cycle stages include multinucleate plasmodia and sporoblasts giving rise to the spores that are described from some species, which occur in the connective tissue of the gills, mantle, and gonad, and are predominately in the digestive gland of bivalves (Ramilo et al., 2018). A strong haemocytic response in those tissues has been previously reported in infected *C. edule* populations (Carballal et al., 2001; Carballal et al., 2020). Although scarce data exist on the impact of haplosporidians on *C. edule* survival, given the strong inflammatory response throughout the tissues, it is likely to be detrimental (Longshaw and Malham, 2013; Carballal et al., 2020). It has been suggested that the dynamics of haplosporidians in their hosts are seasonal and depend on environmental parameters, especially temperature and salinity, which not only impact the vitality and survival of parasites outside their hosts but also influence host-parasite interactions (Arzul and Carnegie, 2015).

Microsporidia, a phylum of fungal-related obligate intracellular parasites, contains species that parasitize almost all types of animals, including other parasites, and all known organ and tissue systems, thanks to their extreme morphological plasticity (Stentiford et al., 2013; Szumowski and Troemel, 2015). Microsporidian pathogens are known hyperparasites of fish and shellfish pathogens (Stentiford et al., 2013), including digeneans and paramyxids infecting *C. edule* populations (Fermer et al., 2009; Fermer et al., 2011b; Villalba et al., 2014). Microsporidian spores invade host cells, where they undergo their entire replicative life cycle and then, differentiate back into spores to return to the environment (Stentiford et al., 2013). The intracellular habitat is fundamental in their ability to evade recognition by the host immune system and to accumulate high parasite burdens within the tissues of infected hosts (Stentiford et al. 2013; Szumowski and Troemel, 2015). Comtet et al., (2003) reported the occurrence of the microsporidian parasite *Steinhausia* sp. in the oocytes of *C. edule* in a natural population in France, where high mortalities occurred. Microsporidians affect host survival but may also influence host phenotype, life history and behaviour (Stentiford et al., 2013).

In turn, bivalves provide a habitat for diverse communities of commensal bacterial microbiota, including various pathogenic and non-pathogenic members of the genus *Vibrio* (Romalde et al., 2014). These bacteria have been repeatedly associated with larval, juvenile and adult bivalves including oysters (Lacoste et al., 2001; Garnier et al., 2007; Le Roux et al., 2016; Azéma et al., 2017; Wang et al., 2021), mussels (Polsenaere et al., 2017; Arab et al., 2021), clams (Paillard et al., 2004b; Beaz-Hidalgo et al., 2010; Schwaner et al., 2020) and scallops (Sainz-Hernandez and Maeda-Martinez, 2005; Rojas et al., 2018; Rojas et al., 2021), and to a lesser extent have been associated with *C. edule* (Paillard et al., 2006; Albuixech-Martí et al., 2021; Garcia et al., 2021). Although bacteria are rarely reported to be the primary cause of disease in adult bivalves (Bower et al., 1994; Lane and Birkbeck, 2000; Prado et al., 2020), they are often associated with high mortalities in shellfish hatcheries (Lacoste et al., 2001; Paillard et al., 2004b; Sainz-Hernandez and Maeda-Martinez, 2005; Dubert et al., 2017). Vibriosis is one of the diseases that may be profoundly affected by climate change. Ocean warming can promote the spread of vibrios and may be the cause of the globally increasing trend in their associated diseases (Vezzulli et al., 2012; Rowley et al., 2014; Zgouridou et al., 2021).

Viruses are by far the most abundant and genetically diverse life forms in the oceans (Breitbart, 2012). Virus-like infections have been identified and associated with mortalities in bivalve hatcheries and wild stocks since the late 1960s (Elston et al., 1992; Elston, 1997; Barber, 2004; Renault and Novoa, 2004; Romalde et al., 2007; Prado-Alvarez et al., 2016; Alfaro et al., 2019). Viruses are likely responsible for many cryptic mortality events, and many proliferative diseases of commercially important marine bivalves including *C. edule*, such as disseminated neoplasia, which is thought to be associated with a viral aetiology (Elston et al., 1992; Barber, 2004; Romalde et al., 2007). In general, mortalities associated with viruses in the marine ecosystem are expected to arise sporadically under increasing physiological stress in the host, causing the host to become immune-compromised and unable to suppress an infection (Suttle, 2007). This has been observed with ostreid herpesvirus-1 (OsHV-1) and variants (in particular, OsHV-1 µVar) in Pacific oyster *Crassostrea gigas* mortality events, which have become more regular and widespread at culture sites (Segarra et al., 2010; Cotter et al., 2010; Prado-Alvarez et al., 2016; Oliver et al., 2019). Moreover, Bookelaar et al. (2020) observed that OsHV-1 µVar may be opportunistic, adapting to new circumstances, such as fewer available susceptible oysters, resulting in a "host jumping" from the natural host to the cohabiting *C. edule*.

Trematode species (Phylum Platyhelminthes) are the most abundant and common macroparasites in coastal waters (de Montaudouin et al., 2009; Magalhães et al., 2018). *C. edule* are hosts to a wide range of trematodes, especially digenean trematodes, that can seriously interfere with host population performance, for instance, reducing growth or fecundity, and causing mortality (Longshaw and Malham, 2013; de Montaudouin et al., 2021; Mahony et al., 2021). Digenean trematodes have complex life cycles involving multiple hosts and free-living larval stages (de Montaudouin et al., 2009; Rowley et al., 2014; Magalhães et al., 2018). Particularly, the species *C. edule* is utilized commonly as either a first or a second intermediate host, and seabird and fish species, that feed on cockles, are the final hosts of these parasites (de Montaudouin et al., 2009; Rowley et al., 2014; Magalhães et al., 2018). In the final hosts, trematodes undergo sexual reproduction followed by the release of eggs in the host's faeces into the environment (Prinz et al., 2009; Magalhães et al., 2018). Free-living stages developed in the environment can then easily infect *C. edule* through their suspension-feeding activity (Magalhães et al., 2017). It is well

known that trematode population dynamics are influenced by environmental factors such as temperature and salinity (Lassalle et al., 2007; Rowley et al., 2014), especially in the free-living stages, as they must face an unpredictable environment on emergence from the host, what may affect their survival and the infection of the next host (Prinz et al., 2009).

This wide range of infectious agents occurring in *C. edule*, their host-parasite interactions, as well as the environmental influence shape a complex pathosystem (Figure 2), which may shed light on how parasites affect directly and indirectly host individual and population health and dynamics, and, in turn, influence the community structure.

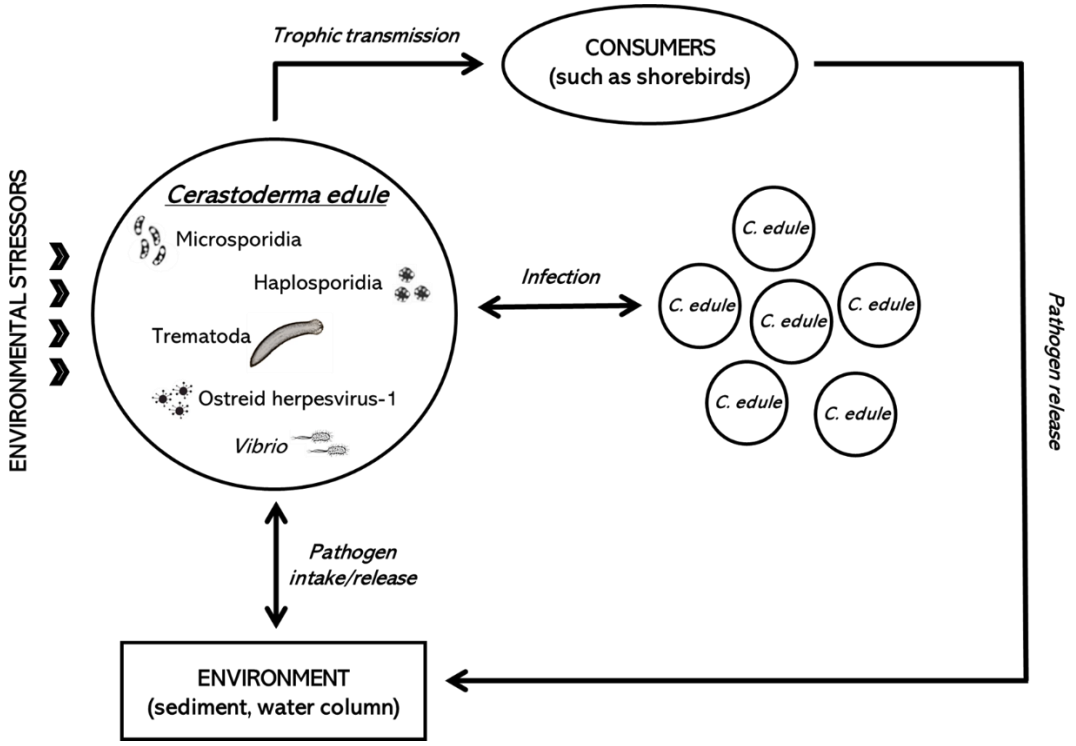

Figure 2: Flow chart illustrating as a model pathosystem the host-parasite dynamics in *Cerastoderma edule* and its interactions with the ecosystem at all levels of ecological organization (individual, population, community, and ecosystem).

2.3. Host-parasite ecology

Parasites are ubiquitous and represent a substantial portion of species diversity and biomass in food webs and ecosystems (Hudson et al., 2006; Poulin, 2010;

Selakovic et al., 2014). Parasites and pathogens live at the expense of the host and divert host energy from growth and reproduction into disease resistance (Selakovic et al., 2014; Egerton et al., 2020). Whether a parasite or pathogen causes disease or not, depends not only on the virulence of the pathogen but also on the host genetics and its immune defences (Mulcahy and Roch, 2009). The immune-competency relationship 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 (Mulcahy and Roch, 2009).

2.3.1. Individual-level effects

In addition to the lethal effects caused by pathogen groups, infectious agents may also cause a broad range of non-lethal effects, and trait-mediated indirect interactions (Figure 3). These may include phenotypic changes in hosts such as: (1) alterations in host physiology, for instance, infected hosts might have different nutrient requirements or metabolic rates than non-infected hosts (Frainer et al., 2018; Magalhães et al., 2020); (2) adjustments in reproductive investment and/or growth rate (Egerton et al., 2020; Mahony et al., 2021); (3) modifications in host morphology, i.e. the phenotypic appearance of the host (Coen and Bishop, 2015); and (4) changes in host behaviour, such as predator or competition avoidance, and/or different habitat preferences (Hatcher, et al., 2006; Frainer et al., 2018). Magalhães et al. (2020) demonstrated that trematode infection exerted a negative effect on the metabolism of *C. edule*, the second intermediate host, by reducing of oxygen consumption rate. Mahony et al. (2021), in turn, observed a reduced proportion of spawning individuals and higher numbers of indeterminate individuals among those *C. edule* infected with trematodes, suggesting trematodes had a deleterious effect on *C. edule* reproduction. These manipulative behaviours in host phenotypes due to the etiological agents, in some instances, represent an adaptation that enhances parasite proliferation and transmission (Poulin, 1995; Thomas et al., 2005). For instance, digenean trematodes may cause the emergence from the substrate or the crawling on the sediment surface of New Zealand cockle *Austrovenus stutchburyi* at soft-sediment shores, making the host more susceptible to predation by avian final hosts, which may considerably enhance the likelihood of successful transmission (Thomas and Poulin, 1998; Leung and Poulin, 2007). However, this hypothesis has been questioned for *C. edule*

(Blanchet et al., 2003; Fermer et al., 2011a). Blanchet et al. (2003), based on both field observations and laboratory experiments, suggested that the bacteria *Pseudomonas fluorescens* could trigger the emergence of *C. edule* from the sediment rather than the coinfecting digenean trematodes, making the cockles more susceptible to predation.

It is also important to stress that not all individuals respond equally to infection. The susceptibility of bivalve molluscs to viral and bacterial infections is generally greater for younger individuals than for adults, which have had more time available to develop defence mechanisms (Lane and Birkbeck, 2000; Renault and Novoa, 2004; Coen and Bishop 2015). Bivalves do not possess acquired immunity, but after encountering microbes as part of their filter-feeder lifestyle, they can respond to infection through their defence cells, the haemocytes, with a certain degree of pathogen specificity (Allam and Raftos, 2015). However, older hosts are more likely to accumulate disease-causing pathogens, as the cumulative time of exposure to parasites is longer than in younger hosts (Breitburg et al., 2015). Thieltges and Reise (2006) observed higher species richness and infection level of metazoan parasites in adult *C. edule* compared to juvenile individuals. The authors related these results in adult cockles to the longer time of exposure to parasites, the cockle size, and the dependency of some parasites on the gonads of mature hosts. The body size in *C. edule* influences the filtration rate and, consequently, the intake rate of parasites (Thieltges and Reise, 2006; Welsh et al., 2019). Therefore, larger *C. edule* will be exposed to larger numbers of infective stages and most likely have higher infection levels (Welsh et al., 2019). Transmission and intensity of infection are correlated with body condition, i.e., individuals that are classed as having a "poor" condition are more likely to be more susceptible to infection (Coen and Bishop, 2015). For instance, the greater reproductive output of larger/older bivalve hosts 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; Coen and Bishop, 2015).

Apart from the direct and indirect effects on their hosts, parasites also interact with other parasites at an individual level. Just like free-living organisms, parasites occupy suitable "habitats" in the host that are limited with regard to the amount of space and resources available (Johnson and Buller, 2011). Coinfections by parasites

occur frequently in many shellfish species and the different parasites that coinfect the same host individual will interact both within the individual host and at a population level (Lassalle et al., 2007; Arzul et al., 2012; Petton et al., 2015; Alfaro et al., 2019; Carella et al., 2020; Albuixech-Martí et al., 2021). Lassalle et al. (2007) reported coinfection with a bacterium (*Vibrio tapetis*), a protozoan (*Perkinsus* sp.), and digenetic trematode metacercariae in *C. edule*. The authors suggested these parasites used different ecological niches within a population, but they had a similar response to environmental factors such as temperature and salinity. Alternatively, a positive association between two *Minchinia* species coinfecting *C. edule* was statistically evidenced in the Irish coastline (Albuixech-Martí et al., 2021). Parasites that infect an already parasitized host are likely to have lower fitness due to the competition or cross-immunity between parasites (Johnson and Buller, 2011). A coinfection could also lead to other benefits by infecting an already parasitized host when the effects of one parasite make the host more vulnerable and easier to exploit for a second parasite. The outcome in this circumstance is parasite coexistence (Johnson and Buller, 2011). Different trematode species in *C. edule* can become "hitchhikers", taking advantage of the infection by metacercariae of *Bucephalus minimus* (Magalhães et al., 2015).

2.3.2. Population-level effects

Parasites have been shown to interfere significantly with the host populations by causing diseases that affect reproduction in complex manners, as well as affecting absolute host population numbers (Coen and Bishop, 2015) (Figure 3). Some infectious agents can increase survival and growth rate, but, in turn, reduce fecundity reaching an energetic trade-off (Selakovic et al., 2014). The trematode *Gymnophallus choledochus* may be responsible for reducing or completely inhibiting the reproduction of *C. edule* through castration to redirect energy from host reproduction to parasite growth, which may have important implications for the population dynamics (Thieltges, 2006). However, negative relationships between the prevalence of diseases and population size and density are not always clear (Coen and Bishop, 2015). For instance, sex ratio distorters are common in microsporidians infecting aquatic hosts (Stentiford et al., 2013). Microsporidia can be transmitted from parent to offspring in a host population and have been associated with the feminisation of the

host males to favour the host reproductive success and, consequently, the parasite transmission (Stentiford et al., 2013).

Parasitism involves a fitness cost for infected hosts and, consequently, there is a selection for hosts with greater resistance to pathogens (Coen and Bishop, 2015). However, selection also acts on the parasites favouring the host infection, thus there may be subsequent selection for parasites with increased virulence and pathogenicity (Coen and Bishop, 2015). The net effect is the co-evolution of the parasite and host, and the coexistence of susceptible and resistant individuals in natural populations (Coen and Bishop, 2015). It has been observed that the frequency of natural disease-resistance in bivalve populations tends to increase following exposure to disease mortality (Carnegie and Burreson, 2011; Lynch et al., 2014). In wild populations, gene flow between disease-affected and refuge areas may also influence the development of disease resistance at the population level (Hofmann et al., 2009).

2.3.3. Community and ecosystem-level effects

In modifying individual and population-level traits of hosts, etiological agents may, directly and indirectly, modify interactions among species (Thomas et al., 1998; Frainer et al., 2018) (Figure 3). The susceptibility and tolerance to parasites vary between host species, depending on the species capacity to bear the infective parasite burden (Hudson et al., 2006; Frainer et al., 2018). Parasites shared by two host species may mediate the host interactions and cause apparent competition between them when levels of infection depend primarily on the rate at which the parasites flow from a tolerant species to a sensitive species, rather than on the density of the sensitive species (Hudson et al., 2006; Hatcher et al., 2012). This can lead to species exclusion, as one tolerant host acts as a reservoir of disease for a more sensitive species (Hudson et al., 2006; Hatcher et al., 2012; Frainer et al., 2018). An example of this is the bacteria *Vibrio tapetis*, the causative agent of brown ring disease (BRD) in clams, that has been also isolated from *C. edule*, which is considered a carrier species of this bacteria since this species of cockle does not exhibit brown ring disease symptoms in the wild (Lassalle et al., 2007). Alternatively, parasitism can ameliorate the deleterious effects of one host on another, enhancing the coexistence of two host species (Hatcher et al., 2012; Frainer et al., 2018). This happens when a dominant competitor or consumer is more strongly regulated by the parasite (e.g., density-dependent transmission or

reducing fitness differences), which creates an advantage for rare species, allowing the persistence of inferior competitors in the communities and maintaining species diversity (Hudson et al., 2006; Poulin, 2010; Hatcher et al., 2012; Frainer et al., 2018). Infectious agents may also influence the outcome of biological invasions through enemy release or biotic resistance if they differentially affect native and non-native species (Coen and Bishop, 2015; Goedknegt et al., 2017; Costello et al., 2021). Goedknegt et al. (2017) observed spillover of the introduced red worm *Mytilicola orientalis* (native to Japan) from introduced Pacific oyster *C. gigas* to native *C. edule*.

Predator-prey interactions are also important in structuring communities and food-web structure and may similarly be influenced by disease. There is increasing evidence regarding the importance of trait-mediated indirect interactions and their significant influence on foraging behaviour and predation rates (Hatcher and Dunn, 2011; Selakovic et al., 2014; Frainer et al., 2018; Anaya-Rojas et al., 2019). Some parasites can alter the phenotype or the behaviour of the intermediate host (e.g., impaired mobility, altered microhabitat choice, or altered body colouration) to make the host more susceptible to predation and improve transmission success, which has favoured the evolution of some remarkable manipulation strategies (Poulin, 1995; Leung and Poulin, 2007; Leung and Poulin, 2011). As well as influencing existing predator-prey relationships, parasites may facilitate new trophic interactions (Coen and Bishop, 2015). Le Grand et al. (2010) related the unexplained presence of *C. edule* at the surface of the sediment in Arcachon Bay with the higher prevalence and intensity of disseminated neoplasia in them compared to those buried normally. The stranded individuals, thus, are more accessible to the predators. The shells of stranded cockles, in turn, create a habitat for a rich and distinctive epibiont community. In addition, the cockles on the sediment surface reduce the sediment bioturbation, which can boost the abundance and richness of certain benthic invertebrates (Mouritsen and Poulin, 2005; Selakovic et al., 2014).

Those parasite effects on the host species may, therefore, trigger parasite-mediated trophic cascades (Frainer et al., 2018; Anaya-Rojas et al., 2019). Microsporidian parasites are also well-known for playing a significant role in exacerbating or suppressing diseases (Stentiford et al., 2013; Gleason et al., 2014). O'Grady et al. (2015) suggested, based on ecological modelling, that the

hyperparasitism by *Unikaryon legeri* on the trematode *Meiogymnophallus minutus* may enable a higher abundance of *C. edule* (host of the trematodes), assuming that the trematodes negatively impact on cockles and their survival, and considering that the hyperparasite is detrimental to trematodes. Thereby, the greater abundance of *C. edule* in the estuarine ecosystem could be beneficial for their consumers, such as birds, fish and crustaceans. Likewise, the greater abundance of *C. edule* could enhance sediment bioturbation and, consequently, the dynamics and stability of the sediment. The infection may, thus, occur at a low trophic level and influence consumers at upper trophic levels by regulating the prey or resource availability (Hatcher et al., 2012; Frainer et al., 2018). Likewise, a top-down trophic cascade may be induced by parasites of a high trophic level, by releasing the immediately lower trophic level from predation or consumption pressure (Hatcher et al., 2012; Frainer et al., 2018). Infection may also influence species at mid-trophic levels, possibly leading to changes in species populations of the same functional group or propagating its effect up and down trophic levels triggering trophic cascades (Selakovic et al., 2014). For instance, Stentiford et al., (2017) observed that *Hyperspora aquatica* (Microsporidia) may have exploited the parasitic lifestyle of their paramyxean and digenean hosts to 'hitch-hike' between hosts of differing trophic positions, such as bivalve molluscs and susceptible crustacean hosts. By directly and indirectly affecting trophic relationships, parasites can be key determinants of food web structure and energy flow (Coen and Bishop, 2015; Frainer et al., 2018). For instance, trematode infection can negatively affect the metabolism of *C. edule*, reducing *C. edule* secondary production and, consequently, influencing the energy transfer in coastal ecosystems and the structure of the ecosystem functioning (Magalhães et al., 2020).

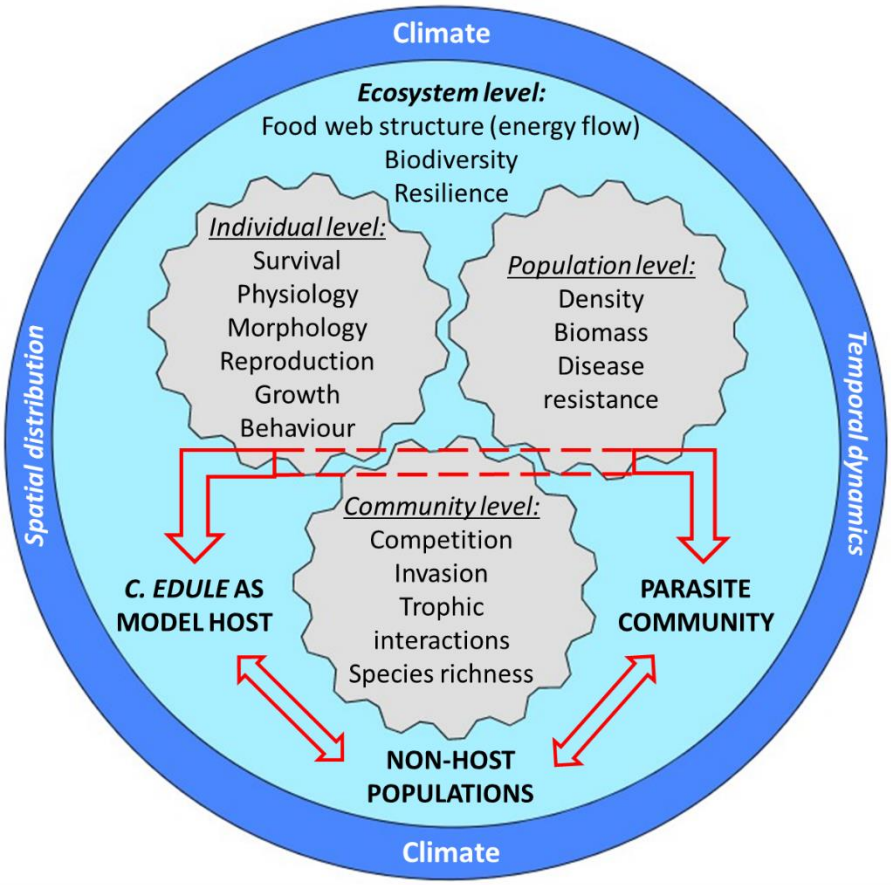

Figure 3: Representation of the host-parasite interplay in *Cerastoderma edule* and its interaction with non-host populations within the natural environment, and a summary of the parasite-mediated effects at the individual, population, community, and ecosystem levels.

Therefore, parasites may not only significantly reduce the host fitness and abundance in the wild but also affect population processes and shape community structure (Hudson et al., 2006; Lafferty and Harvell, 2014; Coen and Bishop, 2015; Frainer et al., 2018). Because of their potential influence on driving biodiversity, ecosystem functioning and resilience, there is growing evidence that a diversity of parasites can be a sign of ecosystem health (Hudson et al., 2006; Frainer et al., 2018). Likewise, given that many parasites are host specific, a diverse and abundant community of parasites might be reflective of a diverse and abundant community of hosts (Hudson et al., 2006).

2.4. Parasite transmission

2.4.1. Transmission routes used by parasites

Parasite transmission is a key process in host-parasite interactions. Direct transmission occurs in parasite species that are spread among conspecific bivalve hosts, either through horizontal transmission between individuals or vertical transmission from parent to offspring (Coen and Bishop, 2015). Parasites can also be trophically transmitted via predator-prey interactions (Coen and Bishop, 2015). Parasite consumption may affect food web structure, energy transfer and disease dispersal, because of the high productivity of parasites, their unique nutritional composition, and their pathogenicity in hosts (Johnson et al., 2010). Norris (1999) observed that oystercatchers *Haematopus ostralegus* feeding on *C. edule* do not consume the larger more energetically profitable individuals, usually associated with higher helminth parasites intensity, even though consuming these preys would increase their rate of energy intake. However, there is a trade-off between energy intake and exposure to helminth parasites in the selection of the prey (Norris, 1999). Concomitant predation is the most common way for parasites to become prey, which occurs when an infected host is eaten by a predator (Johnson et al., 2010) (Figure 4). Recently, Fu et al. (2019) demonstrated that the predation of molluscs and zooplankton by migratory birds played an essential role in the dissemination of *Vibrio parahaemolyticus* and *Vibrio mimicus* in the estuary of the Liaohe River in China. Some complex life cycle parasites induce changes in host characteristics, such as physical appearance, stamina or behaviour, that increase the likelihood of host consumption (Johnson et al., 2010; Hatcher et al., 2012). However, parasite manipulation might also result in ingestion by unsuitable consumers, in which case parasites are digested along with prey (Johnson et al., 2010; Hatcher et al., 2012).

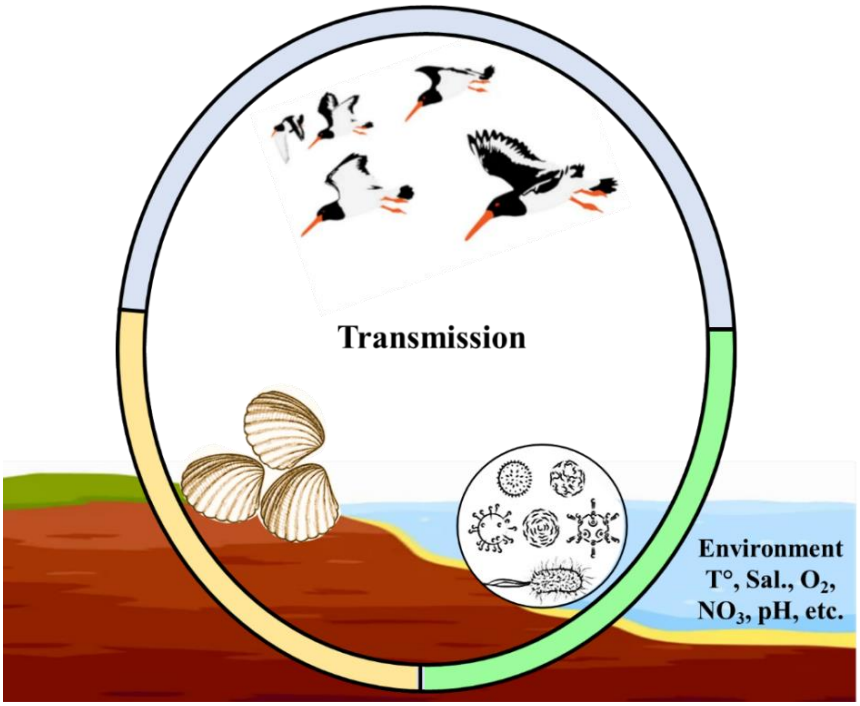

Figure 4: Representation of the disease connectivity and trophic transmission including different compartments: *Cerastoderma edule*, shorebirds as their consumers, and the physical environment (sediment and water column) related to some potential environmental stressors for the host-parasite system.

2.4.2. Role of non-host species in the parasite transmission

Non-host species may interfere with parasite transmission when they actively or passively prey upon free-living infectious stages of parasites and lead to reduced infection levels in the target host (Prinz et al., 2009; Johnson and Thieltges, 2010; Hatcher et al., 2012). Such predation effects can occur in many parasites with free-living stages, and it is increasingly proposed as an important regulatory mechanism for many diseases (Thieltges et al., 2008; Welsh et al., 2014). For instance, Brachyuran crabs (shore crab *Carcinus maenas*) and common shrimp (*Crangon crangon*) can actively prey on free-living cercariae of the trematode *Himasthla elongata* that infect *C. edule* as an intermediate host (Thieltges et al., 2008). Subsequently, Welsh et al. (2014) confirmed by experiments that other intertidal organisms such as the amphipod *Gammarus marinus*, barnacle *Semibalanus balanoides*, oyster *C. gigas*, brush-clawed shore crab *Hemigrapsus takanoi* and marine isopod *Idotea balthica*, can also reduce the number of cercariae and interfere with the transmission of *H. elongata*. Thus, the

presence of free-living stages of parasites in the water column not only affects the parasite consumption but can also induce trophic cascades, releasing the specific hosts from mortality induced by parasites (Ben-Horin et al., 2015).

Despite the current knowledge of how the host and non-host populations interfere with parasite transmission, the understanding of these processes and the interactions between hosts and non-hosts is not entirely clear. For instance, ostreid herpesvirus-1 (OsHV-1), herpes-like viruses infecting oysters as their natural hosts (Lynch et al., 2012), have been detected in nineteen other bivalve species including clams (Renault et al., 2001), cockles (Evans et al., 2017; Bookelaar et al., 2020), scallops (Arzul et al., 2001) mussels (O'Reilly et al., 2018), and also in *C. maenas* (Bookelaar et al., 2018). Although herpesviruses are well known for causing high mortalities in *C. gigas*, to date, it is unknown whether other bivalve species, such as *C. edule*, are only passive carriers of OsHV-1 and/or have a potential to be affected by viral infections leading to high mortalities (Bookelaar et al., 2020).

2.4.3. Host density influence on parasite transmission

Host density can have both positive and negative influences on parasite transmission. High host densities associated with directly transmissible diseases may increase disease prevalence and intensity by increasing contact between infected and uninfected individuals (Anderson and May, 1991; Coen and Bishop, 2015). However, parasites with multiple hosts might have a negative relationship between host density and infection (Coen and Bishop, 2015). A reduction in parasite loads at high host densities has been observed in *C. edule* populations, where the dispersion of infective stages of trematodes across the population resulted in lower infection intensities of individual cockles (Magalhães et al., 2017). Epidemiological models predict that many parasites require a threshold host population size, below which the parasite will inevitably become extinct (Hatcher et al., 2012). This was tested by O'Grady et al. (2015) with a threshold model of parasitism using a system of hyperparasitized trematodes *M. minutus* which infects *C. edule*. The authors concluded that a threshold population of trematodes within a *C. edule* individual is needed to provide a sufficient environment for the hyperparasite population to grow and persist.

Moreover, specialist parasites are particularly vulnerable to secondary extinction following the demise of their host (Hatcher et al., 2012), while parasites that have a complex life cycle are more likely to encounter new host species than directly transmitted parasites (Stentiford et al., 2013). Alternatively, although host extinction from parasitism alone appears rare, it is still a matter of concern, because parasitism can reduce numbers in a population to a size where there is an increased extinction risk from all the stochastic processes that affect small populations (Hudson et al., 2006; Selakovic et al., 2014).

Parasite transmission is affected not only by the host immune system and population density, but also by multiple biological and environmental drivers. Therefore, an ecosystem approach should be taken in order to achieve a more holistic understanding of the factors leading the parasite transmission.

2.5. Associated environmental stressors on host-parasite dynamics

Environmental conditions potentially influence disease processes through differential effects on pathogens and hosts (Lafferty and Holt, 2003; Soon and Zheng, 2020; Zgouridou et al., 2021). Conditions that compromise host responses to pathogens can result in increased numbers of infected individuals and the proliferation of parasites within hosts (Breitburg et al., 2015). Furthermore, parasites might reduce the tolerance threshold of host individuals to extreme environmental conditions such as oxygen depletion, extreme temperatures, and salinity fluctuations (Macey et al., 2008; Longshaw and Malham, 2013; Zgouridou et al., 2021). Consequently, parasite burdens that are considered harmless in favourable environmental conditions could become stressful harmful agents in unfavourable environmental conditions (Macey et al., 2008; Longshaw and Malham, 2013; Zgouridou et al., 2021). In general, stressed individuals should be more susceptible to infection if exposed (Lafferty and Holt, 2003). However, the stressor could simultaneously reduce opportunities for infection by reducing host density, making it more likely that parasites will be extirpated by stress upon the host (Lafferty and Holt, 2003).

For host-parasite systems in estuarine environments, abiotic factors (e.g., temperature, salinity, dissolved oxygen, etc.) are particularly critical in shaping the

host-parasite interactions (Gallana et al., 2013; Coen and Bishop, 2015; Yu et al., 2021). Intertidal organisms like *C. edule*, which are exposed to low tide, are further exposed to desiccation, changes in salinity and temperature extremes (Gosling, 2004). Even though some bivalve species are adapted to cope temporarily with these stressors, if the stressors approach critical values this adaptive response fails, causing severe physiological stress and consequent mortality (Gosling, 2004). *C. edule* is able to respond to rhythmic changes in the tidal flow, however, any dramatic alterations in salinity can only be tolerated for short periods before mortalities are induced (Nossier, 1986). Likewise, mortality events of *C. edule* throughout their range have been related to extreme low winter temperatures (Malham et al., 2012). It has been also observed that *C. edule* at the high shore may be more susceptible to infections since it experiences more stressful abiotic conditions such as air exposure, fluctuating temperatures, variable tidal strengths, precipitation, etc. (Wegeberg and Jensen, 2003). Likewise, it has been suggested that *C. edule* at the high shore are more likely to be in contact with other host species (Lynch et al., 2014).

Temperature is one of the most important factors determining physiological processes, ecological patterns, and the limits on the geographic distribution of bivalves, especially in intertidal habitats more exposed to temperature changes (Verdelhos et al., 2015a). Temperature can affect hosts and parasites asymmetrically, leading to antagonistic and synergistic interactions among the temperature responses of hosts and parasites that determine the net consequences of temperature changes on disease dynamics (Kirk et al., 2018). It is well-known that the shedding of infective stages and the success of transmission of trematode cercariae between bivalve hosts, *C. edule* among them, is more fruitful as temperatures increase (Lassalle et al., 2007; Rowley et al., 2014; Zgouridou et al., 2021). Upper temperatures have been also associated with a higher prevalence of haplosporidians in *C. edule* (Carballal et al., 2020). Warming in coastal regions may, for instance, result in increased bacterial growth and intensification of virulence in *Vibrio* species in shellfish populations, including *C. edule* (Rowley et al., 2014). This may lead to periodic epizootics of vibriosis in the summer months, especially in shallow water, estuaries, and the intertidal environment (Rowley et al., 2014). In turn, *C. edule* are sensitive to extreme temperatures, which make them more vulnerable to diseases under thermal stress (Rowley et al. 2014). Moreover, when the temperature rises, oxygen levels decrease

and metabolic rates increase, suggesting that warming could lead to respiratory stress, thus increasing the organism's susceptibility to disease (Lafferty et al., 2004; Breitburg et al., 2015). Reduction in oxygen content can potentially lead to anoxia within the water and the sediment, which will have a major influence on benthic fauna, especially on sessile organisms and infauna, such as cockles, with no ability to move (Elliott and de Jonge, 2002).

Salinity is also a major factor impacting estuarine bivalves, which are often exposed to short-term (tidal) and long-term (rain periods) changes in salinity (Verdelhos et al., 2015b). This stress often results in behavioural and physiological responses and, ultimately, it may lead to mortality episodes, frequently associated with salinity declines, but also to high salinity values during drought periods (Verdelhos et al., 2015b). Allam and Raftos (2015) observed that shifting salinity gradients may result in changes to host-parasite relationships in bivalve populations by altering the overlap between parasite and host distributions, changing the abundance of parasites and/or hosts and influencing immune systems. Low salinities may depress the immune system of the hosts such as molluscs, but do not negatively affect parasites and, consequently, increase the incidence of disease (Coen and Bishop, 2015). Nevertheless, there are also examples where the pathogens are limited by the low salinity reducing the infection success, as was observed with the haplosporidian *Minchinia* in *C. edule* in Ireland (Albuixech-Martí et al., 2020).

Changes in environmental conditions can, thus, alter the landscape patterns of host-parasite distributions and interactions, and influence both spatial and temporal patterns and the severity of epizootics (Breitburg et al., 2015; Soon and Zheng, 2020; Zgouridou et al., 2021).

2.6. Conclusions

There are still many gaps in the understanding of the disease-mediated effects and the processes that define host-parasite systems in the current changing marine environment. However, studies related to pathosystems and their ecological dynamics in aquatic ecosystems are scarce. Therefore, this review was intended to shed light on ongoing changes in *C. edule* populations and their broader ecological communities

when considering the impacts of infectious agents and the diseases they cause. The rise of marine epizootics and their increased risk of occurrence due to climate change and other anthropogenic disturbances make necessary an intensification of our understanding of the epizootic dynamics, especially in vulnerable habitats such as intertidal and mudflats environments. In this regard, *C. edule* may be an ideal model organism to develop our knowledge within the natural framework, as has been proved in this review. Therefore, a horizon scanning approach for the epizootic and emergent pathogens within *C. edule* may provide an early warning of potential emergence and outbreaks of previously non-problematic infectious agents. As this review emphasised, it is essential to move towards the study of more complex host-pathogen systems and their relationship with the environment in order to have a better understanding of the infectious agents as part of the ecosystem. Further research with an ecosystem approach will not only be important for the sustainability of *C. edule* but also for wild and cultured bivalve populations and the functions of nearshore ecosystems. An integrative approach considering the infectious agents as integral components of all ecosystems would provide a better local scale understanding of the distribution and abundance of hosts and infectious agents and the ways in which they interact within the environment, as has been highlighted in this review.

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CHAPTER 3

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

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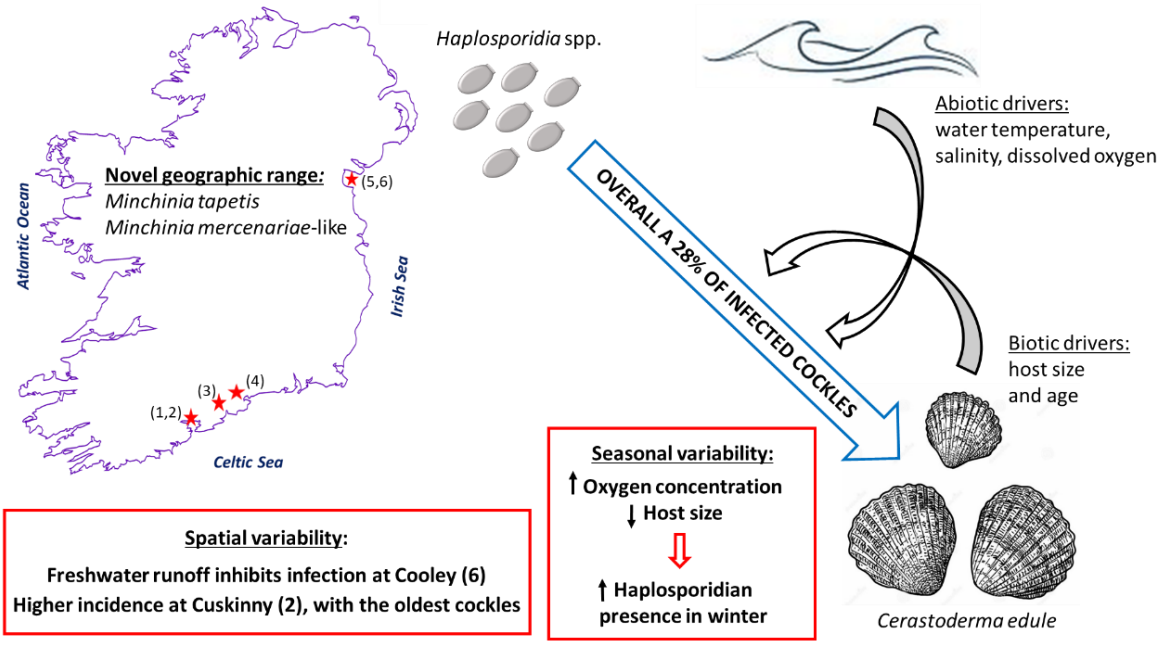
Abstract

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 temporal variability 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 with 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 temporal variability of emergent 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.

3.1. 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 is 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 the 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, a 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) temporal variability 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 temporal variability of parasite occurrence to relevant environmental parameters (temperature, salinity and dissolved oxygen in water) and biotic factors (cockle size and age).

3.2. Materials and Methods

3.2.1. Sampling

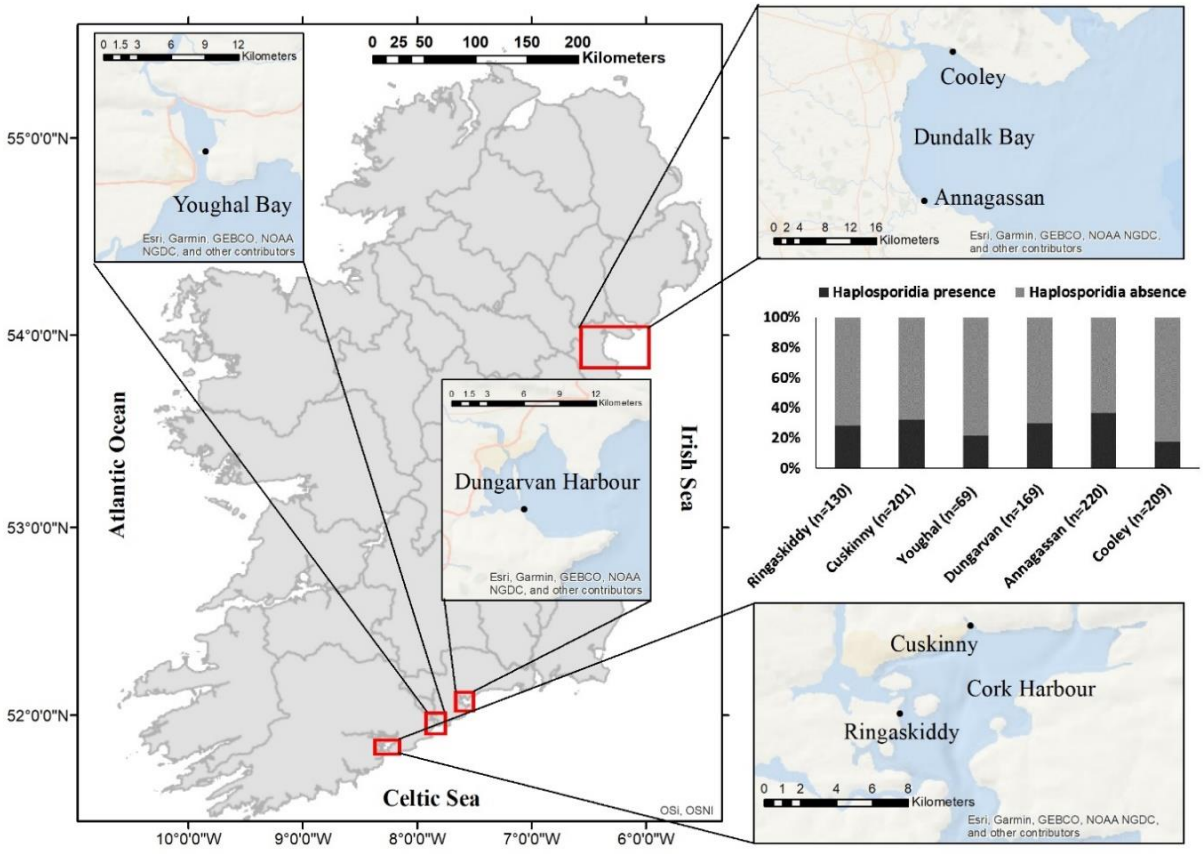


Figure 1: Map of Ireland highlighting the cockle sample sites and the mean haplosporidian presence and absence (%) at each sample site (Ringaskiddy and Cuskinny in Cork Harbour; Youghal Bay; Dungarvan Harbour; Annagassan, and Cooley in Dundalk Bay).

Cockles were sampled (from April/July 2018 to April 2019) within the commercial cockle 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 cockle 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).

Table 1: Sample site description (www.npws.ie/protected-sites/spa) including water quality rating (Water Quality in 2017- An Indicators Report, EPA) 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 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

3.2.2. Sample processing and diagnostic methods

3.2.2.1. 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).

3.2.2.2. 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.

3.2.2.3. 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).

The quantity and the quality of the extracted DNA from a subsample (n=120) of the collected cockles were established using NanoDrop 1000 Spectrophotometer to avoid the diagnosis of false negatives due to low DNA quantity and/or DNA of poor quality. DNA purity was assessed by determining the ratio of spectrophotometric absorbance of each extracted sample at 260/280 nm (A260/A280 ratio; an indicator of protein or phenol contamination). Pure DNA extraction should have an A260/A280 ratio ~1.8, higher values indicate the presence of RNA along with DNA.

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. Purified

DNA of *C. edule* was used as a positive control. Two positive controls were included in each PCR assay to confirm that the amplification worked. Likewise, negative controls (two replicates with ddH_2O) were included in each PCR assay to confirm the absence of contamination. 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. Purified DNA of the pathogen was used as a positive control. Two positive controls were included in each PCR assay to confirm that the amplification worked. Likewise, negative controls (two replicates with ddH_2O) were included in each PCR assay to confirm the absence of contamination. Electrophoresis of the amplification products was conducted in a 2% agarose gel. The expected amplified product (amplicon) size for generic haplosporidian was 350bp.

3.2.2.4. 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 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, which was 0.2µl, and the annealing temperature which was 60°C. Purified haplosporidian DNA was used as a positive control. Two positive controls were included in each PCR assay to confirm that the amplification worked. Likewise, negative controls (two replicates with ddH_2O) were included in each PCR assay to confirm the absence of contamination. 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	350	*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

3.2.3. 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 the 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.

3.2.4. 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 temporal 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 was 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 et al., 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 ΔAIC values ≤ 2 (i.e. not statistically different from the best performing model with ΔAIC = 0) (Thomas, 2017).

3.3. Results

3.3.1. Cockle speciation and identification of haplosporidian species

The examination of the quantity and quality of the extracted DNA from a subsample (n=120) of the collected cockles revealed that all tested samples showed a good DNA quantity, with a mean quantity of 304.13 ng/µl (range between 76.35 ng/µl and 1305.47 ng/µl). Among the subsample, 90% (n=108/120) of the samples had a

DNA quantity higher than 100 ng/µl. Likewise, all the tested samples showed high-quality DNA, with a mean of A260/A280 = 1.77 and a range between 1.6 and 2.02.

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; Suppl. Figure 1).

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 of 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 to 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 the 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).

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 number of PCR-positive cases (%) for each parasite at each site and season

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.

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 in order to confirm the presence of haplosporidians in those individuals. Multinucleate plasmodia and haplosporidian spores were observed in those sections along with low numbers of uninucleate haplosporidian cells (Figure 2e, 2f), supporting the molecular screening results. No obvious differences in host pathology (haplosporidian morphology, etc.) were observed between those individuals (Figure 2e, 2f) and the individuals that were positive for *Minchinia*-specific PCRs (Figure 2a, 2b, 2c, 2d), 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.

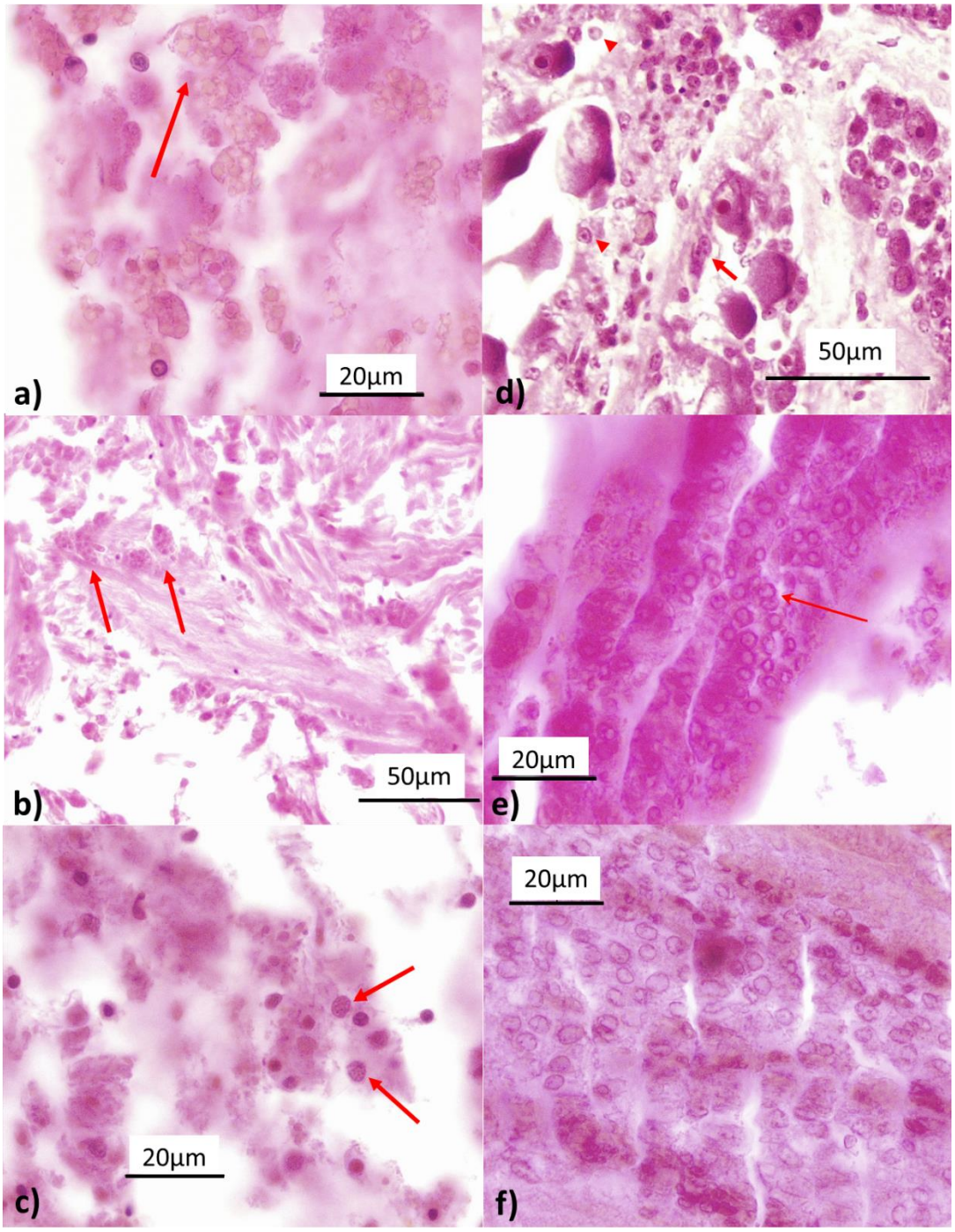


Figure 2: a) Multiple haplosporidia-like sporonts in the connective tissues of *Cerastoderma edule*; b-c) multinucleate plasmodia in the connective tissue of *C. edule*; d) multinucleate plasmodia (arrow) along with uninucleate haplosporidian cells (arrowheads) in the connective tissue of *C. edule*; and e-f) a large number of haplosporidian spores in the mantle tissue of *C. edule*.

3.3.2. *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 temporal variability (Full_Model, AIC = 1154.2) had a better fit than the binomial GLMM with only the temporal 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.

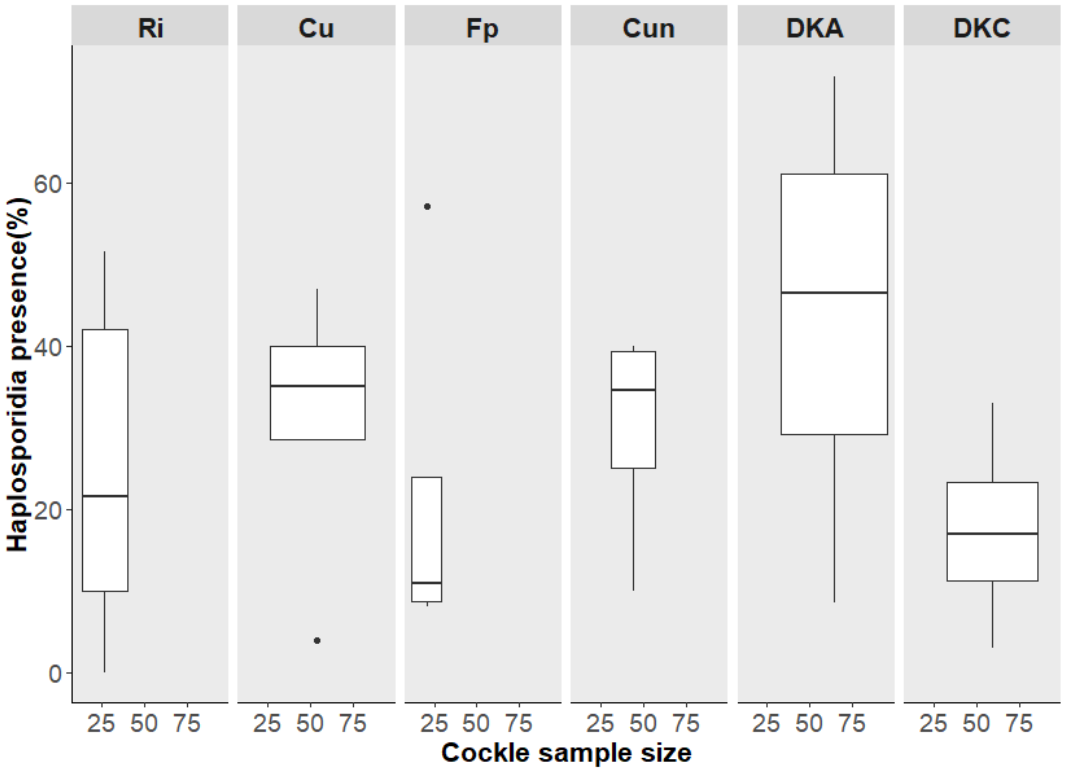


Figure 3: Haplosporidian distribution in cockles collected at each sample site (Ri: Ringaskiddy and Cu: Cuskinny, nested sites in Cork Harbour; Fp: Youghal Bay; Cun: Dungarvan Harbour; DKA: Annagassan and DKC: Cooley, nested sites in Dundalk Bay), combining the data from all the seasons. The boxplot 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.

3.3.3. *Temporal variability in the haplosporidian presence*

Although haplosporidians infecting *Cerastoderma edule* were found throughout the year, temporal variability 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 temporal 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 temporal variability had a significant effect on shaping the haplosporidian distribution through the sites.

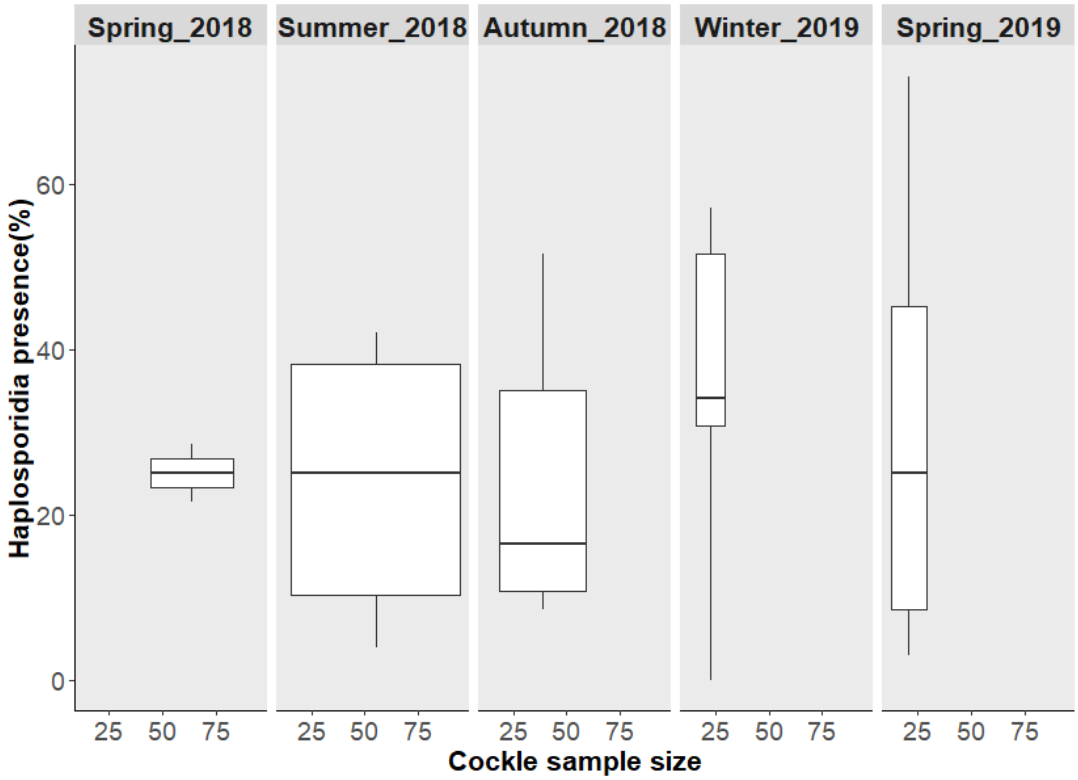


Figure 4: Haplosporidian distribution in cockles collected from different seasons (spring 2018, summer 2018, autumn 2018, winter 2018/19, spring 2019), combining the data from all the sample sites. The boxplot compactly displays the distribution of the response variable - Haplosporidia presence (%) -. 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.

3.3.4. *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 a 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			

The 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, $\Delta AIC \geq 2$, 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			

The 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 ($\Delta AIC \geq 2$) and, consequently, less plausible.

3.4. 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 in 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, however, the presence of haplosporidian cells in those individuals was confirmed by histology. Given the good quantity and high-quality of extracted DNA present in the samples, 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 highly divergent genes that characterise this parasite group hinder the accurate identification of the different species (Hartikainen et al., 2014). There are still many haplosporidian species whose DNA sequence has not been described yet, and numerous non-identified haplosporidians continue to be reported from many different invertebrate hosts and habitats (Burreson and Ford, 2004; Hartikainen et al., 2014). 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.

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 the 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 the fact that 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 at Ringaskiddy possibly indicating a lower *C. edule* density or that a mortality event may have occurred at that site, which was also suggested by the younger cockles sampled at Ringaskiddy compared to Cuskinny. While the environmental conditions were similar at both beds of Cork Harbour, the cockle size at Cuskinny was larger 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 the 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 better knowledge of emergent 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 harvested and non-harvested *Cerastoderma edule* populations.

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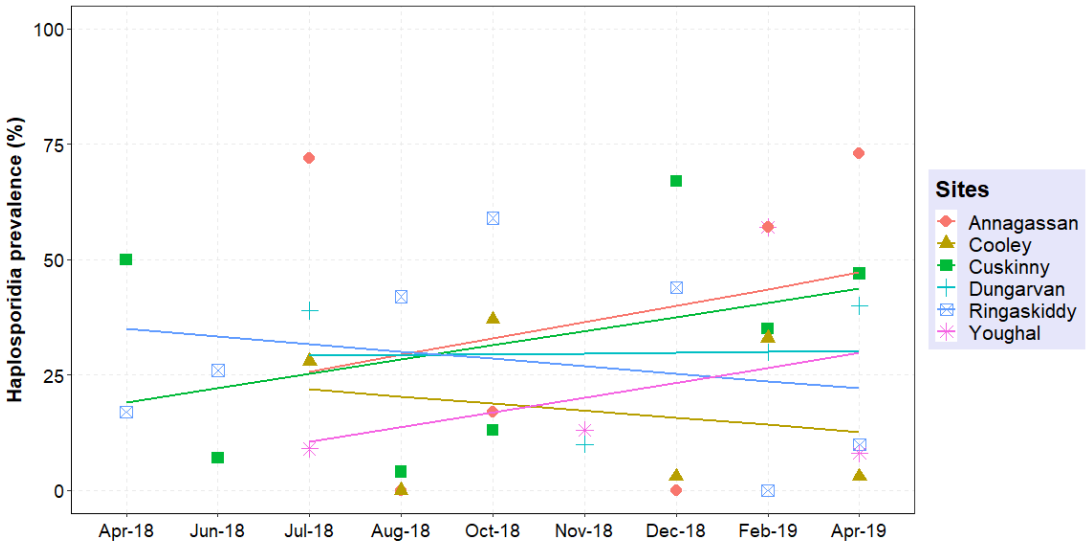
Supplementary Material



Suppl. Figure 1: Total haplosporidian prevalence (%) in *C. edule* from the different sampling months (April 2018, June 2018, July 2018, August 2018, October 2018, November 2018, December 2018, February 2019, April 2019) at the different sample sites, showing the regression trend line of the parasite distribution at each site.

CHAPTER 4

Co-occurrence of pathogen assemblages in a keystone species the common cockle *Cerastoderma edule* on the Irish coast

Co-occurrence of pathogen assemblages in a keystone species the common cockle *Cerastoderma edule* on the Irish coast

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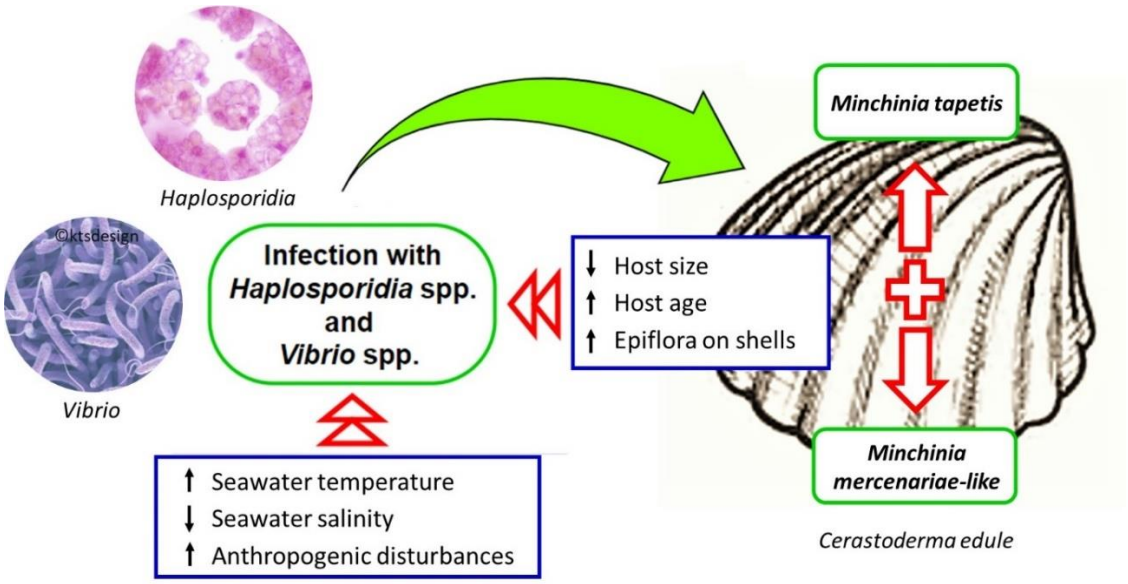
Abstract

Despite coinfections being recognised as the rule in animal populations, most studies focus on single pathogen systems. Pathogen interaction networks and the drivers of such associations are lacking in disease ecology studies. Common cockle *Cerastoderma edule* populations are exposed to a great diversity of pathogens, thus making them a good model system to investigate. This study examined the diversity and prevalence of pathogens from different taxonomic levels in wild and fished *C. edule* on the Irish coast. Potential interactions were tested focussing on abiotic (seawater temperature and salinity) and biotic (cockle size and age, and epiflora on shells) factors. No Microsporidia nor OsHV-1µVar were detected. Single infections with Haplosporidia (37.7%) or *Vibrio* (25.3%) were more common than two-pathogen coinfected individuals (9.5%), as expected by random chance. Fished *C. edule* populations with high cockle densities were more exposed to infections. Higher temperature and presence of epiflora on cockle shells promoted coinfection in warmer months. Low seawater salinity, host condition and proximity to other infected host species influenced coinfection distribution. Moreover, a positive association between two *Minchinia* species was observed, most likely due to their different pathogenic effect and use of resources within the host, as seen in previous studies. However, natural variation in the presence and prevalence of multiple pathogens can be expected at the microscale sampling frame within the cockle beds. Therefore, further research is needed in order to achieve a better understanding of the potential associations occurring between pathogens and the responsible underlying mechanisms. Overall,

findings highlight the major influence that ecological factors have on pathogen interactions and host-pathogen interplay.

Keywords: cockle health, coinfection, Haplosporidia, *Vibrio*, pathogen interactions, confounding factors.

Graphical abstract:



Highlights:

- The study demonstrated the presence and interdependence of coinfecting pathogens in *Cerastoderma edule*.
- The risk of coinfection may have been promoted by higher temperature and the presence of epiflora on the cockle shells in warmer months.

- Site influence in the coinfection distribution might be due to lower seawater salinity, host condition and proximity to other infected host species.
- Fished *C. edule* populations with high cockle densities might have been more exposed to coinfection.
- A positive two-way association between *Minchinia tapetis* and *Minchinia mercenariae*-like organisms was demonstrated.

4.1. Introduction

Animal hosts are exposed to complex pathogen assemblages that ultimately form a dynamic community within them (Johnson and Buller, 2011). However, research into host-pathogen interactions remains dominated by the study of one host-one pathogen systems despite the fact that hosts can simultaneously carry several infectious agents, with consequences for the dynamics of each agent and host health. Coinfections have been previously reported in shellfish hosts: including *Vibrio tapetis*, *Perkinsus* sp. and digenetic trematodes in cockles (Lassalle et al., 2007); ostreid herpesvirus-1 (OsHV-1) and *Vibrio* spp. in oysters (Petton et al., 2015; Alfaro et al., 2019); and protozoans and bacteria in clams (Arzul et al., 2012; Carella et al., 2020); and in some of the cases with a dramatic effect on disease susceptibility (Cox, 2001; Lassalle et al., 2007; Arzul et al., 2012; Alfaro et al., 2019). Processes at different scales of ecological organization from the within-host level (e.g., interactions of coinfecting parasites) to the ecosystem level (e.g., the influence of environmental variables) across space and over time have a key influence on complex natural systems (Hellard et al., 2015). Therefore, moving from one host-one pathogen systems towards an ecosystem view of host-pathogen interactions and their ecology is essential.

Detection of interspecific parasite/pathogen interactions in natural populations is not easy, due to complex networks of indirect effects making it difficult to infer underlying processes (Hellard et al., 2012). Interactions among coinfecting infectious agents can, in fact, alter host pathology, parasite transmission and virulence evolution, also influencing the spread of infections at a population level resulting in disease outbreaks, as described previously in bivalves (Lassalle et al., 2007; Arzul et al., 2012; Alfaro et al., 2019). These effects can be the opposite, i.e. coinfections within the host may reduce infection success yet still enhance pathology or, in contrast, co-occurring parasites/pathogens may weaken host pathology increasing the infection success (Johnson and Hoverman, 2012). For example, mortality induced by one parasite can also eliminate the availability of hosts for other parasites (Jolles et al., 2008), likewise, morbidity induced by one parasite can increase exposure to a second, even if within-host interactions are antagonistic (Karvonen et al., 2009).

Simultaneous infections may therefore trigger a whole spectrum of outcomes within the hosts, both synergistic and antagonistic, i.e. the weight of one or both

infectious agents may be suppressed (Fukami et al., 2010), one or both agents may be amplified resulting in coexistence (Thomas et al., 1998), or one may be amplified and the other suppressed (Johnson and Hoverman, 2012). Parasite/pathogen interactions can be not only direct, such as competition for attachment sites, competition for host resources, and predation upon one another (intra-guild predation) (Hatcher et al., 2006; Johnson et al., 2010; Hatcher et al., 2012), but interactions can also be indirect or host-mediated often involving changes in immunity such as cross-immunity (apparent competition) and immune suppression (apparent facilitation) (Jolles et al., 2008). For instance, Johnson and Hoverman (2012) suggested cross-reactive immunity as a cause of a decrease in parasite persistence in their experiments with a group of related larval trematodes in Pacific chorus frogs (*Pseudacris regilla*). Johansen and Sommer (2001) suggested that the immune suppression triggered by a primary infection could be the cause of multiple bacterial infections in Atlantic salmon (*Salmo salar*).

The co-occurrence of infectious agents may also result simply because the same risk factors promote their presence and not because the different pathogen groups are interacting synergistically (Vaumourin et al., 2015). Risk factors shared by two or more infectious agents can occur at the level of the host individual (e.g. host condition and age), population (e.g. host density) or landscape (e.g. climatic factors), increasing the probability of infection by both/all agents (Hellard et al., 2015). Such confounding factors may influence host exposure and/or host susceptibility promoting simultaneous infections (Vaumourin et al., 2015). Environmental parameters such as seawater temperature and salinity, host density or host physiological conditions and behaviour are common confounding factors that may influence the co-occurrence of different pathogen groups and the epidemiological and geographic patterns of infection and disease (Hellard et al., 2012; Vaumourin et al., 2015). Confounding factors must be considered in field studies since those factors can create statistical associations between infectious agents even if there is no true biological interaction between them (Kuris and Lafferty, 1994).

Generalist host species, such as the common cockle *Cerastoderma edule*, with wide spatial distributions, are exposed to a large range of environmental conditions, and a greater diversity of parasites and pathogens, and consequently are more likely to be coinfected (Vaumourin et al., 2015; Mahony et al., 2020; de Montaudouin et al.,

2021), 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., 2010; de Montaudouin et al., 2021), but may also be infected by a range of pathogens (Rowley et al., 2014; de Montaudouin et al., 2021), providing a microcosm for the parasite/pathogen community. Within this community, there are haplosporidian protists such as the genus *Minchinia* (Engelsma et al., 2011; Longshaw and Malham, 2013; Ramilo et al., 2018; Lynch et al., 2020b; Albuixech-Martí et al., 2020; de Montaudouin et al., 2021), numerous members of the bacterial family *Vibrio* (Carballal et al., 2001; Lassalle et al., 2007; de Montaudouin et al., 2021), viruses such as ostreid herpesvirus-1 microVar (OsHV-1 µVar) (Carballal et al., 2003; Evans et al., 2017; Bookelaar et al., 2020; de Montaudouin et al., 2021) and microsporidian species reported in digeneans and paramyxids infecting cockle populations (Goater, 1993; Fermer et al., 2009; Fermer et al., 2011; Villalba et al., 2014; de Montaudouin et al., 2021). Cockles are a keystone species responsible for different ecosystem services, i.e. carbon storage, energy cycling, food source for seabirds, etc., being also good sentinel and bioindicator species (Malham et al., 2012; Carss et al., 2020). Moreover, the common cockle is one of the main non-cultured bivalve species harvested in western European waters, with a high economic value (Rowley et al., 2014; Carss et al., 2020). Consequently, cockles have been well-studied and are useful model species. Given the complex parasite/pathogen assemblages within the cockles and their intricate interactions and effects, a comprehensive and integrated approach that considers not only the synergistic impact of coinfections but also the role of ecological factors driving the infection across space and over time is needed.

Future climate scenarios predict a warming marine environment and increased precipitation events resulting in pulse events of reduced/fluctuating salinity in nearshore ecosystems (Allam and Raftos, 2015; Coen and Bishop, 2015). Consequently, climate change may be expected to have a significant effect on disease epidemiology (Coen and Bishop, 2015), and may even have significant consequences on zoonoses emergence (Hoarau et al., 2020). In this context, the need for the understanding of ecological processes involved in the transmission and dynamics of infectious agents in host reservoirs is critical (Hoarau et al., 2020). This study,

therefore, aims to assess the interactions between infectious agents, their hosts, and the environment.

For that purpose, this study examined the diversity and prevalence of significant pathogen groups, Haplosporidia, *Vibrio* spp., OsHV-1 µVar and Microsporidia, associated with bivalve mortalities globally as well as coinfections and associations of the different infectious agents at an individual and population level in wild and fished *C. edule* along the Irish and Celtic Seas. The role of abiotic (seawater temperature and salinity) and biotic (cockle size and age, and epiflora on their shells) factors in the risk of coinfection throughout the study was also assessed. Developing knowledge on pathogen diversity and co-occurrence in an economically and ecologically important species such as *C. edule* and integrating the role of the environment in the host-pathogen interplay is essential for a better understanding of the interaction networks that can affect disease outcomes and transmissibility in the current changing environment. Findings from this study highlight the complexity of pathogen interactions within a host and the effect of key biotic and environmental drivers shaping disease dynamics, which can also be applied to other coinfected animal host species.

4.2. Materials and Methods

4.2.1. Sampling

Cockles were sampled from spring 2018 to spring 2019 at Cork Harbour (Ringaskiddy and Cuskinny, n=257) on the south coast (Celtic Sea) of Ireland and from summer 2018 to spring 2019 at Youghal Bay (n=69) and Dungarvan Harbour (n=169), with an extensive farming of Pacific oysters (*Crassostrea gigas*), on the south coast (Celtic Sea) of Ireland, where there is no commercial fishing activity (Figure 1). Additionally, 240 cockles were collected from summer 2018 to spring 2019 at the commercial fishery at Dundalk Bay (Annagassan and Cooley) on the northeast coast (Irish Sea) of Ireland (Figure 1). Dundalk Bay is a classified Bivalve Mollusc Production Area and it has supported a commercial dredge cockle (*Cerastoderma edule*) fishery since 2001 (Hervas et al., 2008).

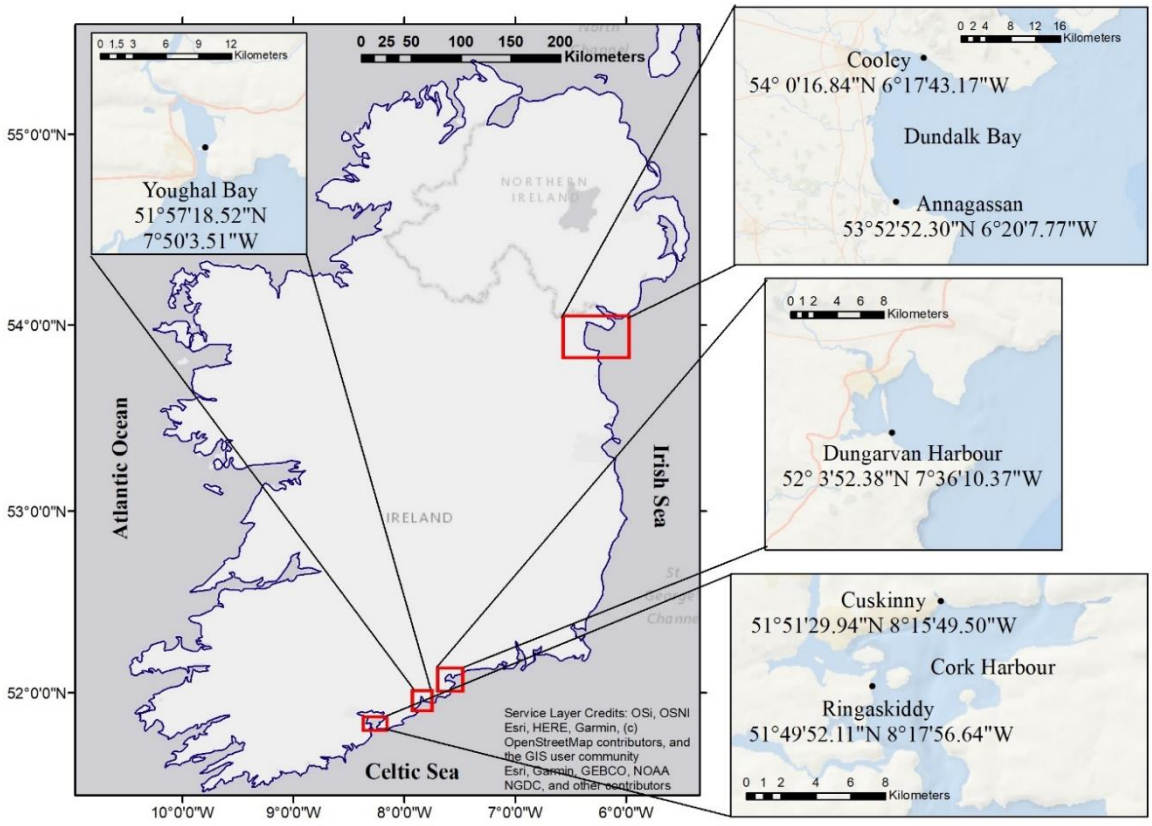


Figure 1: Map of Ireland highlighting the cockle sample sites with coordinates.

The four locations are bays with intertidal sand and mudflats, where a variety of bivalves inhabit. *Cerastoderma edule* densities in the selected sites vary from higher densities, 9.33 ± 3.5 ind/m^2, in Dundalk Bay (Shellfish Stocks and Fisheries Review, Marine Institute and BIM, 2017) to lower densities in the no commercial fishing sites: 1.6 ± 2.1 ind/m^2 in Youghal Bay; 0.8 ± 1.7 ind/m^2 in Cork Harbour; and 0.4 ± 1.3 ind/m^2 in Dungarvan Harbour (Fermer et al., 2011). Cockle mortalities associated with disseminated neoplasia have been previously reported in Cork Harbour (Morgan et al., 2012), while a massive cockle mortality event associated with dinoflagellates occurred in Youghal Bay in the past (Ottway et al., 1979). Dungarvan Harbour has also a history of OsHV-1-associated mortality events (Lynch et al., 2012; Prado-Alvarez et al., 2016). However, to the best of our knowledge, Dundalk Bay has not previously recorded mortality events.

Even though Cork Harbour is an important industrial area and is a busy ferry/shipping terminal, its trophic status (based on the nutrient levels such as phosphorus and nitrogen, growth of algae and dissolved oxygen concentration) was

qualified as unpolluted by the EPA in 2017 along with Dungarvan Harbour, an extremely sheltered bay, while Youghal Bay, close to an urban hub, was classified as intermediate polluted area. In turn, Dundalk Bay showed a localised effect on the trophic status, with Annagassan classified as an unpolluted area and Cooley as an intermediate polluted area, probably due to the influence of water discharge from the local river.

Cockles were collected by hand raking from the intertidal area of each site at spring tide (0.5m-1m) and seasonal intervals. The sample size, outlined in Table 1, was dependant on the availability of cockles at each site and season. Cockles were kept at ambient temperature during the fieldwork and were processed either on the same day or were held out of water overnight at 4°C and were processed the next day.

Table 1: Sample sites, seasons and number of cockles collected.

Sampling dates	Cork Harbour	Youghal Bay	Dungarvan Harbour	Dundalk Bay	Totals
Spring 2018	60	-	-	-	**60**
Summer 2018	39	11	51	60	**161**
Autumn 2018	60	16	58	60	**194**
Winter 2018/19	38	30	30	60	**158**
Spring 2019	60	12	30	60	**162**
Totals*	**257 (34.9%)**	**69 (9.4%)**	**169 (23.0%)**	**240 (32.7 %)**	**735**

(-) No samples collected
** In brackets the % representation of all cockles sampled*

4.2.2. Sample processing and diagnostic methods

4.2.2.1. Morphometrics:

Measurements included (A) cockle body size (height (mm)) of each individual that was recorded using Vernier callipers. The (B) cockle's age was established 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). The presence or absence of epiflora on the shell of each

individual was also recorded. Epiflora was mainly composed of epiphytic algae, although no specific species were identified.

4.2.2.2. Histology:

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

4.2.2.3. Molecular techniques for cockle species identification and pathological screening:

A small piece of gill tissue (2-5mm^2 of tissue) from each cockle was taken and stored at -20°C for molecular assays, to determine the European cockle species (*Cerastoderma edule* (Linnaeus, 1758) or *Cerastoderma glaucum* (Poiret, 1789)) and for subsequent pathological screening. Genomic DNA from the cockle gill tissue was extracted using the chelex-100 extraction method (Walsh et al., 1991; Lynch et al., 2008).

The quantity and the quality of the extracted DNA from a subsample (n=120) of the collected cockles were established using NanoDrop 1000 Spectrophotometer to avoid the diagnosis of false negatives due to low DNA quantity and/or DNA of poor quality. DNA purity was assessed by determining the ratio of spectrophotometric absorbance of each extracted sample at 260/280 nm (A260/A280 ratio; an indicator of protein or phenol contamination). Pure DNA extraction should have an A260/A280 ratio ~1.8, higher values indicate the presence of RNA along with DNA.

Polymerase chain reaction (PCR) was carried out to amplify the nuclear DNA markers *ITS-for* / *ITS Ce-R* / *ITS Cg-R* to differentiate between the presence of *C. edule*, *C. glaucum* species or hybrids (Freire et al., 2011; Table 2). Purified DNA of *C. edule* was used as a positive control. Two positive controls were included in each PCR assay to confirm that the amplification worked. Likewise, negative controls (two

replicates with ddH_2O) were included in each PCR assay to confirm the absence of contamination. Amplification was conducted following the reaction mixture and thermocycling conditions, as well as the visualisation of the product, described in Albuixech-Martí et al. (2020).

Standard PCR screening for haplosporidian detection was carried out in cockle gill tissue samples (n = 735) 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 2). Purified DNA of the pathogen was used as a positive control. Two positive controls were included in each PCR assay to confirm that the amplification worked. Likewise, negative controls (two replicates with ddH_2O) were included in each PCR assay to confirm the absence of contamination. Amplification was conducted following the reaction mixture and thermocycling conditions, as well as the visualisation of the product, described in Albuixech-Martí et al. (2020).

Standard PCR screening for *Minchinia* spp. detection was carried out in the cockle samples that were positive for Haplosporidia by conventional PCR, using specific primers for *Minchinia tapetis* (TAP-For/Rev) and *Minchinia mercenariae*-like (MER-For/Rev) (Albuixech-Martí et al., 2020; Table 2) in the appropriate pairings and separate PCR reactions. Two positive controls (purified DNA of the respective *Minchinia* species) were included in each PCR assay to confirm that the amplification worked. Likewise, negative controls (two replicates with ddH_2O) were included in each PCR assay to confirm the absence of contamination. Amplification was conducted following the reaction mixture and thermocycling conditions, as well as the visualisation of the product, described in Albuixech-Martí et al. (2020).

Standard PCR screening for *Vibrio* spp. in cockle gill tissues was conducted using generic primers Vib-F3/R3, developed in our lab for the detection of *Vibrio* genus (Kett et al., 2022) (Table 2). The screening was conducted in all the collected cockles (n = 735). A total of 2 µl of DNA per individual was used in a total volume of 27.5µl of the reaction mixture containing: 5µl of 5x green buffer, 5µl of dNTPs (1.25mM), 0.5µl of $MgCl_2$, 0.25µl of forward and reverse primers, 0.1µl of GoTaq polymerases, 1.5µl of DMSO and 12.9µl of ddH_2O. Cycling conditions consisted of an initial denaturation of the sample at 95°C for 1 minute followed by 35 cycles of

94°C for 20s, 56°C for 30s, 72°C for 30s and a final elongation at 72°C for 7 minutes. Purified DNA of the pathogen was used as a positive control. Two positive controls were included in each PCR assay to confirm that the amplification worked. Likewise, negative controls (two replicates with ddH_2O) were included in each PCR assay to confirm the absence of contamination. Electrophoresis of the amplification products was conducted in a 2% agarose gel. The expected amplified product (amplicon) size for *Vibrio* spp. was 286bp.

Standard PCR screening for ostreid herpesvirus-1 (OsHV-1) and variants, including OsHV-1 µVar, was carried out in cockle gill tissue samples (n = 735) using specific primers OHVA/OHVB that amplify small products of the *ORF4* gene in the OsHV-1 virus and its variants (Lynch et al., 2013; Table 2). Purified DNA of the pathogen was used as a positive control. Two positive controls were included in each PCR assay to confirm that the amplification worked. Likewise, negative controls (two replicates with ddH_2O) were included in each PCR assay to confirm the absence of contamination. Amplification was conducted following the reaction mixture and thermocycling conditions described in Lynch et al. (2013). Electrophoresis of the amplification products was conducted in a 2% agarose gel. The expected amplified product (amplicon) size for OsHV-1 and variants was 385bp.

Standard PCR using specific primers MicIF1/MicIR1 and MicIF2/MicIR2 (Lynch et al., unpublished data confirmed to be specific to Microsporidia spp. by Sanger sequencing; Table 2) to detect two unidentified microsporidian species was carried out in cockle gill tissue samples (n = 735). Purified microsporidian DNA was used as a positive control. Two positive controls were included in each PCR assay to confirm that the amplification worked. Likewise, negative controls (two replicates with ddH_2O) were included in each PCR assay to confirm the absence of contamination. Both reaction mixture and thermocycling conditions were the same as used with the PCR for OsHV-1 and variants, as well as the visualisation of the product. The expected amplified product (amplicon) size for microsporidian spp. was 180bp.

Table 2: Description of PCR primer pairs 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	350	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
Vib-F3 Vib-R3	CAA CAG AAG AAG CAC CGG CT	CAC GCT TTC GCA TCT GAG TG	286	*Vibrio* spp.
OHVA OHVB	TGC TGG CTG ATG TGA TGG CTT TGG	GGA TAT GGA GCT GCG GCG CT	385	*Ostreid herpesvirus-1 and variants*
MicIF1 MicIR1	GTG GAC GCT AGT CTC ACA GGT T	TTG CAC CAG AAG GTT TAC GAC ACA T	180	*Microsporidia sp.1*
MicIF2 MicIR2	ATG CAT GCG TAA GCG AAG CAG TTA T	TCT CTT GCA CCA GAA GGT TTA CGA C	180	*Microsporidia sp. 2*

Quantitative PCR (qPCR) for detection and quantification of *Vibrio aestuarianus* in the cockle samples that were positive for *Vibrio* spp. was performed using specific primers (Table 3) for detection of the molecular chaperone *dnaJ* gene, following the protocol of McCleary and Henshilwood (2015). Negative template controls consisting of 5 µl of double-distilled water (ddH_2O) were included in triplicates in each run. 5 µl of *V. aestuarianus* DNA suspension was used in triplicates as positive controls in each run. All samples were performed in triplicates using 5 µl of DNA. C_t (Cycle threshold) values were used to determine PCR quantification and detection limits by a Thermo Hybaid PCR express thermal cycler. A tested sample

was considered positive if its mean C_t value was below 37. Six prepared 1:10 serially diluted *V. aestuarianus* DNA standards were tested in triplicates as stated in McCleary and Henshilwood (2015). Standard curves aim at checking the efficacy of the PCR reaction and at estimating the number of bacterial DNA copies present in tested samples. Results were interpreted by linear regression for slope analysis, and the PCR amplification efficiency (E) was calculated according to: $E = [10^{1/(-slope)}] - 1$ (McCleary and Henshilwood, 2015). Absolute quantitation for DNA copies was carried out by comparing the C_t obtained against the standard curve with known copy numbers.

Viral load for ostreid herpesvirus-1 and variants was investigated by qPCR with a small number of individuals (n=30, whose standard PCR results displayed some smear in the visualisation of the product) to verify that there was no viral load by carrying out a more specific screening and increasing the amount of DNA processed. The protocol of Pepin et al. (2008) was followed, using the specific primers HVDP-F/HVDP-R (Table 3). Each run included two positive DNA controls (genomic OsHV-1 DNA) and two blank controls (ddH_2O). All samples were conducted in duplicates using 5 µl of DNA. C_t values were used to determine PCR quantification and detection limits by a Thermo Hybaid PCR express thermal cycler. Amounts of viral DNA (copies/µl) are calculated by comparison with standard curve values obtained from amplification reactions carried out with serial dilutions of the genomic OsHV-1 DNA. Absolute quantitation for OsHV-1 DNA copies is carried out by comparing the C_t obtained against the standard curve with known copy numbers. PCR efficiency ($E=10^{(-1/slope)} - 1$) is determined by drawing standard curves from a serial dilution's analysis of genomic OsHV-1 DNA to ensure that E ranged from 95% to 105% (Pepin et al., 2008).

Table 3: Description of qPCR primer pairs and probe.

Primers	**Sequence**	**Specificity**
dnaJ f420	GTGAAGGGACGGGTGCTAAG	*Vibrio aestuarianus*
dnaJ r456	CCATGACAAGTGCCACAAGTCT	
dnaJ p441 (probe)	FAM-AGGGCACGTCGGC-MGB	
HVDP-F	ATTGATGATGTGGATAATCTGTG	*Ostreid herpesvirus-1 and variants*
HVDP-R	GGTAAATACCATTGGTCTTGTTCC	

Direct sequencing was carried out on representative PCR products (n=10) amplified using generic *Vibrio* Vib-F3/R3 primers (Kett et al., 2022) to identify the *Vibrio* species present in the samples. Likewise, direct sequencing was carried out on representative PCR products (n=5) 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 beyond the *Minchinia* species screened. Genomic DNA from selected individuals 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 (Source Bioscience). Each sequence was matched against the National Center for Biotechnology Information (NCBI) nucleotide database with BLASTn (Basic Local Alignment Search Tool), which finds regions of local similarity between sequences to identify and confirm the DNA being detected in the PCRs.

4.2.3. Environmental data

This study was conducted using E.U. Copernicus Marine Service Information (marine.copernicus.eu/services-portfolio/access-to-products). Two environmental parameters critical in shaping host-pathogen interactions in estuarine and marine environments (Coen and Bishop, 2015) were selected for this study as potential confounding factors: seawater temperature and salinity. Recorded data in seawater temperature (°C) and salinity (PSU), 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) along the Irish coast.

4.2.4. Statistical analysis

Statistical analysis was performed in R version 3.2.3. statistical software. The pathogen occurrence was modelled as pathogen presence/absence in each cockle for each pathogen group and their combinations, and it was used in the subsequent analysis to examine the associations between the different pathogen groups present through the samples.

Pearson's Chi-squared tests between the observed values and the expected values of the prevalence of coinfected individuals were conducted to assess whether

the total observed values were significantly different (p(Chisq) < 0.05) from the expected prevalence of coinfections or were consistent with random chance (p(Chisq) > 0.05).

Association screening approach (SCN), described in detail by Vaumourin et al. (2014), was conducted by running 5000 simulations (NS) with the presence/absence of Haplosporidia and *Vibrio* spp. to determine a potential association between these two pathogen groups, under the null hypothesis of random pathogen associations (p(SCN) < 0.05). Subsequently, a second SCN (NS=5000) was performed with the presence/absence of the different pathogen species detected and quantified in the samples, i.e. *Minchinia tapetis*, *M. mercenariae*-like and *Vibrio aestuarianus*.

Corrected Pearson's Chi-square test, described in detail by Hellard et al. (2012), was applied to the Haplosporidia and *Vibrio* spp. presence/absence data to determine if the coinfection was due to an underlying biological interaction or to confounding factors considered (seawater temperature, salinity, cockle size and age, and epiflora on their shells). Likewise, the test was applied to the *Minchinia tapetis* and *M. mercenariae*-like presence/absence data. Biological interactions between two pathogens, therefore, will be suspected when the probability of coinfection is not random once confounding factors have been considered (p(Corr χ^2) < 0.05). In the model, running 1000 bootstraps, the effects of the confounding factors are summarised as F1+F2+F3+F4+F5.

Moreover, Pearson's Chi-squared tests were used to determine whether the prevalence observed of single infections and two-pathogen coinfections were significantly different (p(Chisq) < 0.05) among the sample sites and throughout the year. Fisher's exact test was conducted when the frequencies of the pathogen occurrence in a site or season were < 4, to gain accuracy.

4.3. Results

Table 4: Prevalence (%, positive individuals/screened individuals) of Haplosporidia spp., *Minchinia tapetis*, *M. mercenariae*-like, *Vibrio* spp. and *Vibrio aestuarianus* screening in *Cerastoderma edule* 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 and Cuskinny	Haplosporidia spp.	**45.0% (27/60)***	15.4% (6/39)	41.7% (25/60)	21.1% (8/38)	28.3% (17/60)	32.3% (83/257)
	Minchinia tapetis	15.0% (9/60)	7.7% (3/39)	**18.3% (11/60)***	2.6% (1/38)	1.7% (1/60)	9.7% (25/257)
	Minchinia mercenariae-like	**23.3% (14/60)***	0% (0/39)	8.3% (5/60)	0% (0/38)	10.0% (6/60)	9.7% (25/257)
	Vibrio spp.**	13.3% (8/60)	12.8% (5/39)	**15.0% (9/60)***	5.3% (2/38)	11.7% (7/60)	12.1% (31/257)
	V.aestuarianus	**1.7% (1/60)***	0% (0/39)	0% (0/60)	0% (0/38)	**1.7% (1/60)***	0.8% (2/257)
Youghal Bay	Haplosporidia spp.	-	9.1% (1/11)	12.5% (2/16)	**56.7% (17/30)***	8.33% (1/12)	30.4% (21/69)
	Minchinia tapetis	-	0% (0/11)	0% (0/16)	0% (0/30)	0% (0/12)	0% (0/69)
	Minchinia mercenariae-like	-	0% (0/11)	**12.5% (2/16)***	3.3% (1/30)	0% (0/12)	4.3% (3/69)
	Vibrio spp.**	-	27.3% (3/11)	**43.8% (7/16)***	16.7% (5/30)	33.3% (4/12)	27.5% (19/69)
	V.aestuarianus	-	**27.3% (3/11)***	6.3% (1/16)	0% (0/30)	0% (0/12)	5.8% (4/69)
Dungarvan Harbour	Haplosporidia spp.	-	**39.2% (20/51)***	10.3% (6/58)	30% (9/30)	40% (12/30)	27.8% (47/169)
	Minchinia tapetis	-	**2.0% (1/51)***	0% (0/58)	0% (0/30)	0% (0/30)	0.6% (1/169)
	Minchinia mercenariae-like	-	0% (0/51)	0% (0/58)	0% (0/30)	**10.0% (3/30)***	1.8% (3/169)
	Vibrio spp.**	-	**56.9% (29/51)***	**56.9% (33/58)***	6.7% (2/30)	36.7% (11/30)	44.4% (75/169)
	V.aestuarianus	-	**43.1% (22/51)***	0% (0/58)	0% (0/30)	3.3% (1/30)	13.6% (23/169)
Dundalk Bay-Annagassan and Cooley	Haplosporidia spp.	-	**98.3% (59/60)***	28.3% (17/60)	45.0% (27/60)	38.3% (23/60)	52.5% (126/240)
	Minchinia tapetis	-	**70.0% (42/60)***	23.3% (14/60)	0% (0/60)	0% (0/60)	23.3% (56/240)
	Minchinia mercenariae-like	-	**11.7% (7/60)***	0% (0/60)	1.7% (1/60)	3.3% (2/60)	4.2% (10/240)
	Vibrio spp.**	-	**38.3% (23/60)***	33.3% (20/60)	3.3% (2/60)	26.7% (16/60)	25.4% (61/240)
	V.aestuarianus	-	3.3% (2/60)	**10% (6/60)***	0% (0/60)	0% (0/60)	3.3% (8/240)
Totals by season	Haplosporidia spp.	45.0% (27/60)	**53.4% (86/161)***	25.8% (50/194)	38.6% (61/158)	32.7% (53/162)	37.7% (277/735)
	Minchinia tapetis	15.0% (9/60)	**28.6% (46/161)***	12.9% (25/194)	0.6% (1/158)	0.6% (1/162)	11.2% (82/735)
	Minchinia mercenariae-like	**23.3% (14/60)***	4.3% (7/161)	3.6% (7/194)	1.3% (2/158)	6.8% (11/162)	5.6% (41/735)
	Vibrio spp.**	13.3% (8/60)	**37.3% (60/161)***	35.6% (69/194)	7.0% (11/158)	23.5% (38/162)	25.3% (186/735)
	V.aestuarianus	1.7% (1/60)	**16.8% (27/161)***	3.6% (7/194)	0.0% (0/158)	1.2% (2/162)	5.0% (37/735)

(-) No samples
(*) The highest prevalence (%) for each pathogen group at each site/season is highlighted in bold.
(**) Potential Vibrio species were defined in Table 5

The examination of the quantity and quality of the extracted DNA from a subsample (n=120) of the collected cockles revealed that all tested samples showed a good DNA quantity, with a mean quantity of 304.13 ng/µl (range between 76.35 ng/µl and 1305.47 ng/µl). Among the subsample, 90% (n=108/120) of the samples had a DNA quantity higher than 100 ng/µl. Likewise, all the tested samples showed high-quality DNA, with a mean of A260/A280 = 1.77 and a range between 1.6 and 2.02. Proven that the quantity and quality of extracted DNA were appropriate, subsequent molecular analyses were carried out. In total, 735 cockles were screened to determine the prevalence of infection and coinfections of the target pathogens (Table 4). The cockle speciation PCR confirmed that all the individuals from all sample sites were *Cerastoderma edule*.

4.3.1. Diversity and prevalence of single and multiple infections and the associations between them

No ostreid herpesvirus-1 microVar (OsHV-1 µVar) nor microsporidian species were detected in any of the cockles (n=735) screened by PCR. The only pathogen groups detected during the study were Haplosporidia and *Vibrio*. Overall, Haplosporidia was the most common pathogen found through the samples, with a 37.7% (n=277) occurrence (Figure 2, detailed data in Table 4). Among these 277 infected cockles, two haplosporidian species were identified in the study: *Minchinia tapetis* which was present in 29.6% (n=82/277) of the tested individuals, and *M. mercenariae*-like which was present in 14.8% (n=41/277) (Figure 2, detailed data in 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 for *Minchinia* spp., which could indicate the presence of other haplosporidian species occurring in the samples. Thus, five of those samples amplified using generic HAP-F1/R3 primers were commercially sequenced, however, low-complexity sequences were obtained, and no significant similarity was found in GenBank. Therefore, the 277 positive PCR cases were defined as Haplosporidia spp. for statistical and analysis purposes.

Vibrio was present in 25.3% (n=186) of the total number of cockles screened (Figure 2, detailed data in Table 4). Of the 186 samples that were positive for *Vibrio*, 19.9% (n=37/186) were confirmed by qPCR to be *Vibrio aestuarianus* (Figure 2,

detailed data in Table 4). *V. aestuarianus* positive controls (diluted purified DNA) have a C_t=30 (60 copies of *V. aestuarianus* DNA/µL). Based on that value, 13.5% (n=5/37) of the individuals were classified as heavily infected individuals ($C_t \leq 30$; more than 60 copies of *V. aestuarianus* DNA/µL), while 86.5% (n=32/37) were classified as low-intensity infections ($37 > C_t > 30$; less than 60 copies of *V. aestuarianus* DNA/µL). The PCR efficiency and slope values were also recorded for each standard curve in order to precise accuracy and reproducibility. The mean PCR efficiency value obtained were 100,5% ± 2,72%, and -3,31 ± 0,07% for slope value.

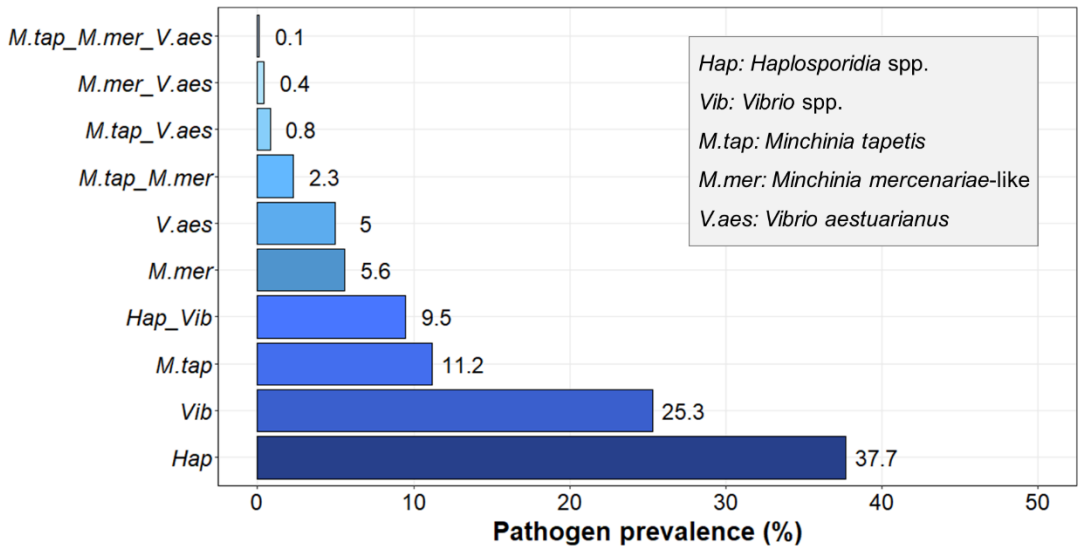


Figure 2: Overall prevalence of infections and coinfections (%) with the different pathogen groups (Hap: Haplosporidia spp.; Vib: *Vibrio* spp.; M.tap: *Minchinia tapetis*; M.mer: *Minchinia mercenariae*-like; V.aes: *Vibrio aestuarianus*).

Direct sequencing was carried out on representative PCR products (n=10) amplified using generic *Vibrio* Vib-F3/R3 primers (Kett et al., 2022) to identify other *Vibrio* species present in the samples apart from *V. aestuarianus*. Using BLASTn, three of the sequenced samples had a query length of 127 to 155bp with an 83 to 91% query coverage and 92.6 to 97.9% percent identity to *Vibrio splendidus* deposited by Gao (2020) (MT445179.1; MT445177.1) and by Landreau et al. (2020) (MT345091.1) (Table 5). Three other PCR product samples that generated sequences had a query length of 82 to 179bp, a query coverage of 78 to 83% and 92.6 to 98.6% percent identity to *Vibrio kanaloae* deposited by Zheng (2020) (MT505700.1) and by

Barcia and Romalde (2020) (CP065151.1; CP065150.1) (Table 5). Another sample, with a query length of 158bp, had 94% query coverage and 98% percent identity to *V. aestuarianus* deposited by Garcia (2018) (MK307696.1) and *Vibrio mediterranei* deposited by Gao et al. (2020) (MT269602.1) (Table 5). Two other samples were identified as *Vibrio* spp., as the blasted sequence was too short (52-69bp) to differentiate robustly between species (Table 5). The 186 positive PCR cases were defined as *Vibrio* spp. for statistical and analysis purposes.

Table 5: Description of the BLASTn results obtained from the sequenced DNA of cockle samples using generic *Vibrio* primers Vib-F3/R3.

Sample site	Season	Species identification	Percent Identity	Query Cover	Query Length (bp)
Youghal Dungarvan Cooley	Summer 2018	*Vibrio spp.* among them: *Vibrio splendidus* and *V. kanaloae*	92.59-98.58%	78-83%	82-179
Annagassan Annagassan	Summer 2018	*Vibrio spp*. most likely *V. splendidus*	97.84-97.92%	88-91%	154-155
Annagassan	Summer 2018	*Vibrio spp.* most likely *V. aestuarianus* or *V. mediterranei*	98.01%	94%	158
Cuskinny Dungarvan	Autumn 2018	*Vibrio spp.*	94.23-94.29%	67-73%	52-69

Being 25.3% (n=186/735) the total prevalence of *Vibrio* spp. and assuming no bias towards coinfection, it would be reasonable to expect 69 of 277 (0.25 × 277) animals infected with Haplosporidia to be coinfected with *Vibrio* spp. Overall, 9.5% (n= 70/735) of the individuals displayed coinfection with the two pathogen groups present in the samples, Haplosporidia and *Vibrio* (Figure 2), while 207 cases of infection with only Haplosporidia were observed. Consequently, the observed coinfection between Haplosporidia and *Vibrio* spp. appears to be coincidental with the expected value ($p(Chisq) > 0.05$).

Moreover, no significant association between these two pathogen groups was displayed when testing by SCN (p-value > 0.05), under the null hypothesis of random pathogen combinations (Table 6), i.e., the pathogen combination was considered not to occur differently than expected by chance.

Table 6: Association screening analysis (SCN) results for all the pathogen combinations between Haplosporidia (HAP) and *Vibrio* (VIB) pathogen groups. The observed (Obs) frequency of each coinfection status is given along with the lower (LL) and upper (UL) limits of the 95% confidence envelope.

Coinfection status	Pathogen groups		Obs	LL	UL	SCN p-value
2-pathogen coinfection	**HAP**	**VIB**	70	51	90	0.5174
1-pathogen infection	**HAP**		207	177	236	0.9832
		VIB	116	92	141	0.5142
Not infected			342	309	376	0.5222

In order to identify the mechanisms driving the pathogen coinfection with Haplosporidia and *Vibrio* pathogen groups, Corrected Pearson's Chi-square Test was also performed including seawater temperature, salinity, cockle height and age, as well as presence/absence of epiflora on the shells (Table 7; Table 8), as confounding factors which may promote pathogen presence. Under the null hypothesis of independence between the two pathogens, the test showed that the observed frequencies for each pathogen combination were not significantly different (Corr χ^2=2.51; ĉ=0.84; p-value 1=0.08; p-value 2=0.08) to the expected frequencies considering the confounding factors as drivers of the infection (Table 9). Therefore, the proportion of double infected individuals can be explained because the same factors promote the pathogen presence and not because the different pathogen groups are interacting synergistically. The differences in the considered environmental and biotic factors between sample sites and throughout the time, thus, might have been driving the risk of infection in *C. edule* populations.

Table 7: Mean ± SD of abiotic (seawater temperature and salinity) and biotic factors (cockle height and age) and the overall percentage of presence of epiflora on the cockle shells at each sample site. The largest value for each factor is highlighted in bold.

Mean ± SD	Cork Harbour	Youghal Bay	Dungarvan Harbour	Dundalk Bay
Temperature (°C)	11.8 ± 2.6	10.7 ± 2.9	**12.2 ± 3.0**	10.8 ± 4.2
Salinity (PSU)	33.6 ± 0.8	**33.9 ± 0.03**	**33.9 ± 0.2**	30.4 ± 0.5
Height (mm)	**29.7 ± 7.8**	27.3 ± 4.8	22.0 ± 4.5	29.6 ± 5.3
Age (growth rings)	**3.6 ± 1.9**	2.9 ± 1.7	1.7 ± 1.0	3.0 ± 1.6
Epiflora (presence/absence)	**10.9%**	7.2%	4.7%	1.7%

Table 8: Mean ± SD of abiotic (seawater temperature and salinity) and biotic factors (cockle height and age) and the overall percentage of presence of epiflora on the cockle shells by season.

Mean ± SD	Spring 2018	Summer 2018	Autumn 2018	Winter 2018/19	Spring 2019
Temperature (°C)	12.2 ± 3.9	12.0 ± 3.3	11.9 ± 3.3	11.5 ± 3.4	11.4 ± 3.4
Salinity (PSU)	32.2 ± 1.6	33.1 ± 1.4	32.9 ± 1.6	32.7 ± 1.7	32.6 ± 1.7
Height (mm)	28.0 ± 7.4	27.2 ± 7.1	27.5 ± 6.9	27.8 ± 6.7	27.9 ± 6.6
Age (growth rings)	2.7 ± 1.7	2.8 ± 1.7	2.8 ± 1.8	2.9 ± 1.8	2.9 ± 1.7
Epiflora (presence/absence)	26.7%	8.7%	2.1%	1.9%	4.9%

Table 9: Observed frequencies of infection with Haplosporidia and *Vibrio* and their combination; and expected frequencies under corrected Pearson's chi-square test considering confounding factors.

Corrected Pearson's Chi-square Test		
Haplosporidia-Vibrio	**Observed frequencies:**	**Expected frequencies:**
-/-	342	351.17
-/+	116	106.83
+/-	207	197.83
+/+	70	79.17

(-/-) Non-infected individuals
(-/+) (+/-) Single infected individuals
(+/+) Double infected individuals

Regarding the specific species quantified in the samples, only 0.8% (n=6/735) of the individuals were coinfected with *Vibrio aestuarianus* and *Minchinia tapetis*, while 0.4% (n=3/735) were coinfected with *V. aestuarianus* and *Minchinia mercenariae*-like (Figure 2). Likewise, 13.8% of the *Minchinia* infected cockles showed a coinfection with both *Minchinia* species (n=17/123), however, the percentage fell to 2.3% when all the screened cockles were considered (n=17/735) (Figure 2). While coinfection with both *Minchinia* species and *V. aestuarianus* was only observed in one of the samples (0.1%, n=1/735) (Figure 2).

Being 5.0% (n=37/735) the total prevalence of *V. aestuarianus* and assuming no bias towards coinfection, it would be reasonable to expect 4 of 82 (0.05 × 82) animals infected with *M. tapetis* to be coinfected with *V. aestuarianus*. Accordingly, the observed prevalence of coinfection with *V. aestuarianus* and *M. tapetis* (n=6/735) was not significantly different to the prevalence expected by random chance (p(Chisq) > 0.05). The observed prevalence of coinfection with *V. aestuarianus* and *M. mercenariae*-like (n=3/735) was also consistent with random chance (p(Chisq) > 0.05) when it was compared to the expected value, i.e., 2 of 41 (0.05 × 41) animals infected with *M. mercenariae*-like would be expected to be coinfected with *V. aestuarianus*. Likewise, 1 of 17 (0.05x17) individuals coinfected with both *Minchinia* species was expected to be coinfected with *V. aestuarianus* and it appears to be coincidental (p(Chisq) > 0.05) to the observed prevalence (n=1/735). However, regarding the coinfection with both *Minchinia* species, being 11.2% (n=82/735) the total prevalence of *M. tapetis*, the observed value of coinfection with *M. mercenariae*-like (n=17/735) was significantly higher (p(Chisq) < 0.001) than expected by random chance, i.e., only 5 of 41 (0.11x41) animals infected with *M. mercenariae*-like would be expected to be coinfected with *M. tapetis*.

Associations between *M. tapetis*, *M. mercenariae*-like and *V. aestuarianus* and all the possible combinations were tested by SCN, revealing a significant association between the two species of *Minchinia* (Table 10). The observed number of coinfected individuals with both *Minchinia* species was significantly overrepresented compared to expected by random chance (p-value < 0.001; Table 10), indicating a significant positive two-way association between these two species. While *M. mercenariae*-like was found to occur alone significantly less often than expected by chance (p-value < 0.05; Table 10).

Table 10: Association screening analysis (SCN) results for all the pathogen combinations between *M. tapetis* (MT), *M. mercenariae*-like (MM) and *V. aestuarianus* (VA). The observed (Obs) frequency of each coinfection status is given along with the lower (LL) and upper (UL) limits of the 95% confidence envelope. Significant associations (p-value < 0.05) are highlighted in bold.

Coinfection status	Pathogen species			Obs	LL	UL	SCN p-value
3-pathogen coinfection	**MT**	**MM**	**VA**	1	0	2	0.4292
2-pathogen coinfection	**MT**	**MM**		16	0	11	**0.0004**
	MT		**VA**	5	0	10	0.7036
		MM	**VA**	2	0	6	0.5568
1-pathogen infection	**MT**			60	53	95	0.0996
		MM		22	21	50	**0.0296**
			VA	29	18	46	0.8016
Not infected				600	558	612	0.1836

The association between *Minchinia* species was further examined by Corrected Pearson's Chi-square Test in order to assess if the biotic and abiotic factors considered in the study (Table 7; Table 8) influenced this coinfection. The test showed that the observed frequencies for each pathogen combination were significantly different from the expected frequencies considering the confounding factors as drivers of the infection (Table 11). The significant outcome (Corr χ^2=3.04; ĉ=0.59; p-value 1=0.02; p-value 2 = 0.02) showed that the two pathogens are not independent of each other, and the proportion of double infected individuals is unlikely to be due only to shared confounding factors.

Table 11: Observed frequencies of infection with *Minchinia tapetis* and *M. mercenariae*-like and their combination; and expected frequencies under corrected Pearson's chi-square test considering confounding factors.

Corrected Pearson's Chi-square Test		
M. tapetis* and *M. mercenariae-like	**Observed frequencies:**	**Expected frequencies:**
-/-	171	166.28
-/+	24	28.72
+/-	65	69.72
+/+	17	12.28

(-/-) Non-infected individuals
(-/+) (+/-) Single infected individuals
(+/+) Double infected individuals

4.3.2. Site influence on single infections and coinfections

Spatial variability was assessed and significant differences (p(Chisq) < 0.05) were found between the sample sites when the prevalence of single infections and two-pathogen coinfections were considered (Figure 3). Dundalk Bay was the site with the highest presence of Haploporidia spp. (52.5%, n=126/240), showing a substantially higher prevalence of *Minchinia tapetis* (23.3%, n=56/240) than the other sites, while no presence of *M. tapetis* was observed in Youghal Bay. Dundalk Bay showed lower seawater salinity and a lower presence of epiflora on the cockle shells than the other sample sites (Table 7). Dungarvan Harbour, with the smallest and youngest cockles collected (Table 7), showed the highest presence of *Vibrio* spp. (44.4%, n=75/169) and a peak of *Vibrio aestuarianus* (13.6%, n=23/169). Moreover, three out of the five individuals with the highest intensity of infection ($C_t \leq 30$) with *V. aestuarianus* were observed at Dungarvan Harbour. Accordingly, the highest prevalence of coinfection with Haplosporidia and *Vibrio* spp. occurred in Dundalk Bay (14.6%, n=35/240) and Dungarvan Harbour (11.8%, n=20/169). However, coinfection with *M. tapetis* and *M. mercenariae*-like was absent in Dungarvan Harbour and Youghal Bay, with Cork Harbour being the site with the highest prevalence (4.3%, n=11/257). The other two-pathogen coinfections with *Minchinia* species and *V. aestuarianus* showed no significant differences between the sites (p(Chisq) > 0.05) since the total number of positive cases detected was too low (*M. tapetis* and *V. aestuarianus* (n=6); *M. mercenariae* and *V. aestuarianus* (n=3)).

Likewise, Cork Harbour was the only site that showed three-pathogen coinfection with both *Minchinia* species and *V. aestuarianus* (0.4%, n=1/257). Cork Harbour, the site with the oldest cockles, had the highest presence of epiflora on the cockle shells compared to the other sample sites (Table 7).

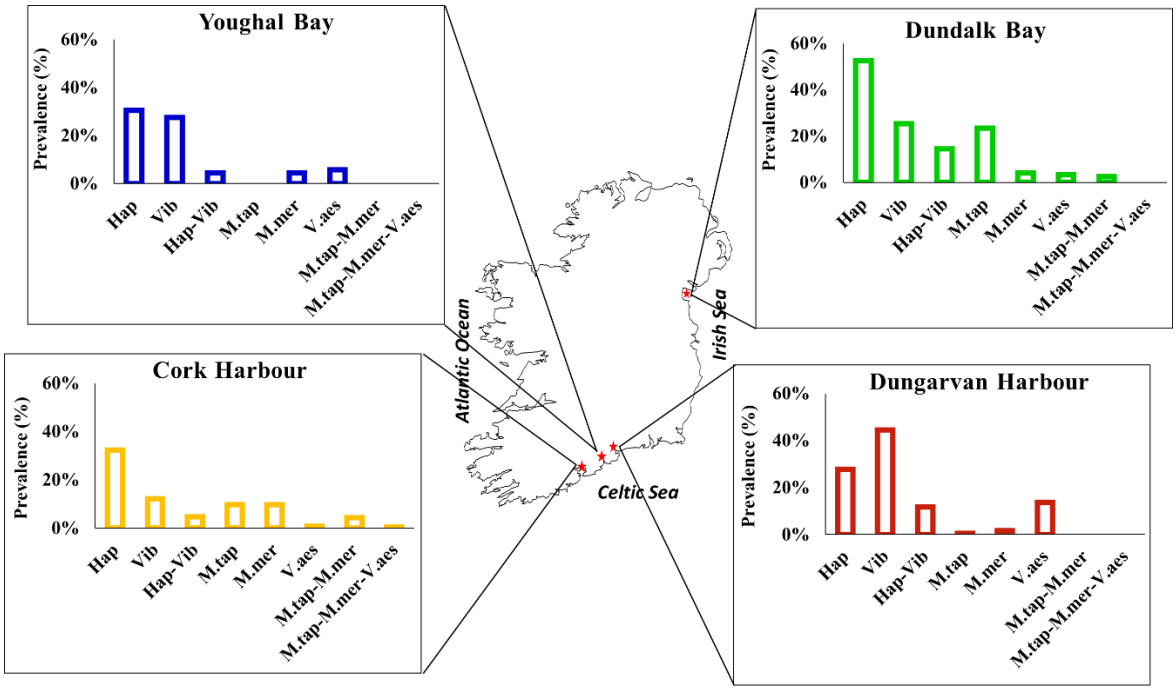


Figure 3: Prevalence (%) of Haplosporidia spp. (Hap), *Vibrio* spp. (Vib), *Minchinia tapetis* (M.tap), *M. mercenariae*-like (M.mer) and *V. aestuarianus* (V.aes) single infections and coinfections (Hap-Vib; M.tap_M.mer; M.tap_M.mer_V.aes) at each sample site.

4.3.3. Temporal impact on single infections and coinfections

Significant temporal variability (p(Chisq) < 0.001) was exhibited when the prevalence of single infections and two-pathogen coinfections were compared between seasons (Figure 4). The absence of *V. aestuarianus* and, in general, a low prevalence of infections were observed in winter 2018/19 (Figure 4), except for Haplosporidia spp. (38.6%, n=61/158). Summer 2018 had the highest prevalence for all pathogens (Figure 4), except for *M. mercenariae*-like which exhibited the highest prevalence in spring 2018 (23.3%, n=14/60). Furthermore, the five individuals with the highest intensity of infection ($C_t \leq 30$) with *V. aestuarianus* were observed during summer 2018. The highest prevalence of coinfection with Haplosporidia and *Vibrio*

spp. occurred in summer 2018 (21.7%, n=35/161), while the highest coinfection prevalence with *M. tapetis* and *M. mercenariae*-like occurred in spring 2018 (11.7%, n=7/60), with no occurrence in winter 2018/19. The higher pathogen incidence during spring and summer corresponded with higher seawater temperature and a peak in the presence of epiflora on the cockle shells (Table 8). The other two-pathogen coinfections with *Minchinia* species and *V. aestuarianus* showed no significant differences throughout the year (p(Chisq) > 0.05) since the total number of positive cases detected was too low (*M. tapetis* and *V. aestuarianus* (n=6); *M. mercenariae* and *V. aestuarianus* (n=3)). There was only one case of three-pathogen coinfection with *M. tapetis, M. mercenariae*-like and *V. aestuarianus* in spring 2019 (0.6%, n=1/162).

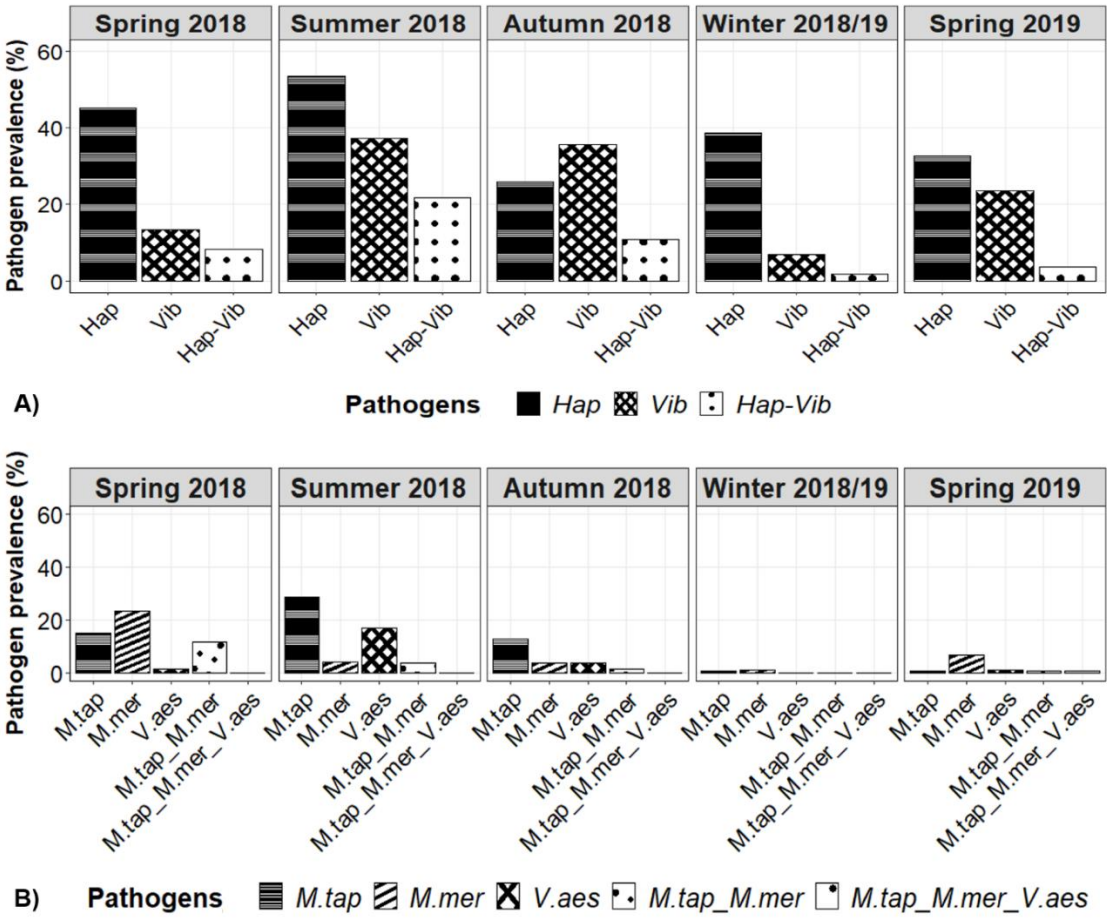


Figure 4: A) Prevalence (%) of Haplosporidia (Hap) and *Vibrio* (Vib) single infections and coinfection (Hap-Vib) by season. B) Prevalence (%) of *Minchinia tapetis* (M.tap), *M. mercenariae*-like (M.mer) and *V. aestuarianus* (V.aes) single infections and coinfections (M.tap_M.mer and M.tap_M.mer_V.aes) by season.

4.3.4. Histopathology

The histological screening was used as a validation method of the PCR and qPCR results. Haplosporidian infection was observed in histological sections of 67 cockles out of a subsample of 70 samples (95.7% visual confirmation) that were positive for generic haplosporidian PCR. Thirty of these histological sections were positive cases for generic haplosporidian PCR but negative for specific *Minchinia* PCRs in order to confirm the presence of haplosporidians in those individuals. No obvious differences in host pathology (haplosporidian morphology, etc.) were observed between those sections (Figure 5b, 5d) and the *Minchinia*-specific positive cases (Figure 5a, 5c). Mostly haplosporidians were observed as multinucleate plasmodia, multiple haplosporidia-like sporonts and developing spores similar to those described for *Minchinia* spp. (Ramilo et al., 2018; Lynch et al., 2020b; Figure 5), and as uninucleate cells stage in lower numbers. Among the sample sites, the haplosporidia-like sporonts were predominantly visualised in the infected individuals from Cork Harbour in spring 2018, autumn 2018 and spring 2019; while the developing spores were observed mainly in the infected individuals from Dundalk Bay in summer 2018 and winter 2018/19. A higher prevalence of haplosporidians was recorded by PCR at both sites. The multinucleate plasmodia were visualised in cockles throughout the year at the different sample sites. The multinucleate plasmodia and haplosporidia-like sporonts were mainly observed throughout the connective tissue of the gills and digestive gland, while the developing spores were principally found in the mantle of infected animals.

Host pathology associated with *Vibrio* species included haemocyte accumulation in the connective tissues, as McCleary and Henshilwood (2015) described in *C. gigas* infected with *V. aestuarianus*. In this study, some of the sections examined (n=28) showed haemocyte accumulation in the connective tissues of *C. edule* (Figure 5). However, the presence of haemocyte infiltrations throughout the organs and tissues has also been previously associated with *Minchinia* infection (Longshaw and Malham, 2013). In this study, only in a minor number of screened samples (n=4), where *Vibrio* and Haplosporidia were detected by PCR, haemocyte infiltrations were observed, all of them at Cork Harbour.

Although ostreid herpesvirus-1 microVar (OsHV-1 µVar) is not visible using histology, cytopathic effects can be observed in infected hosts, such as hypertrophied

cells with a low nucleus-cytoplasm ratio and nuclear abnormalities (Prado-Alvarez et al., 2016). Abnormal cell pathology associated with a viral infection was not observed in the histology (n=70). Similarly, microsporidial spores may be visualised by microscopy in infected hosts (Garcia, 2002), however, it was not observed in the histological sections screened (n=70).

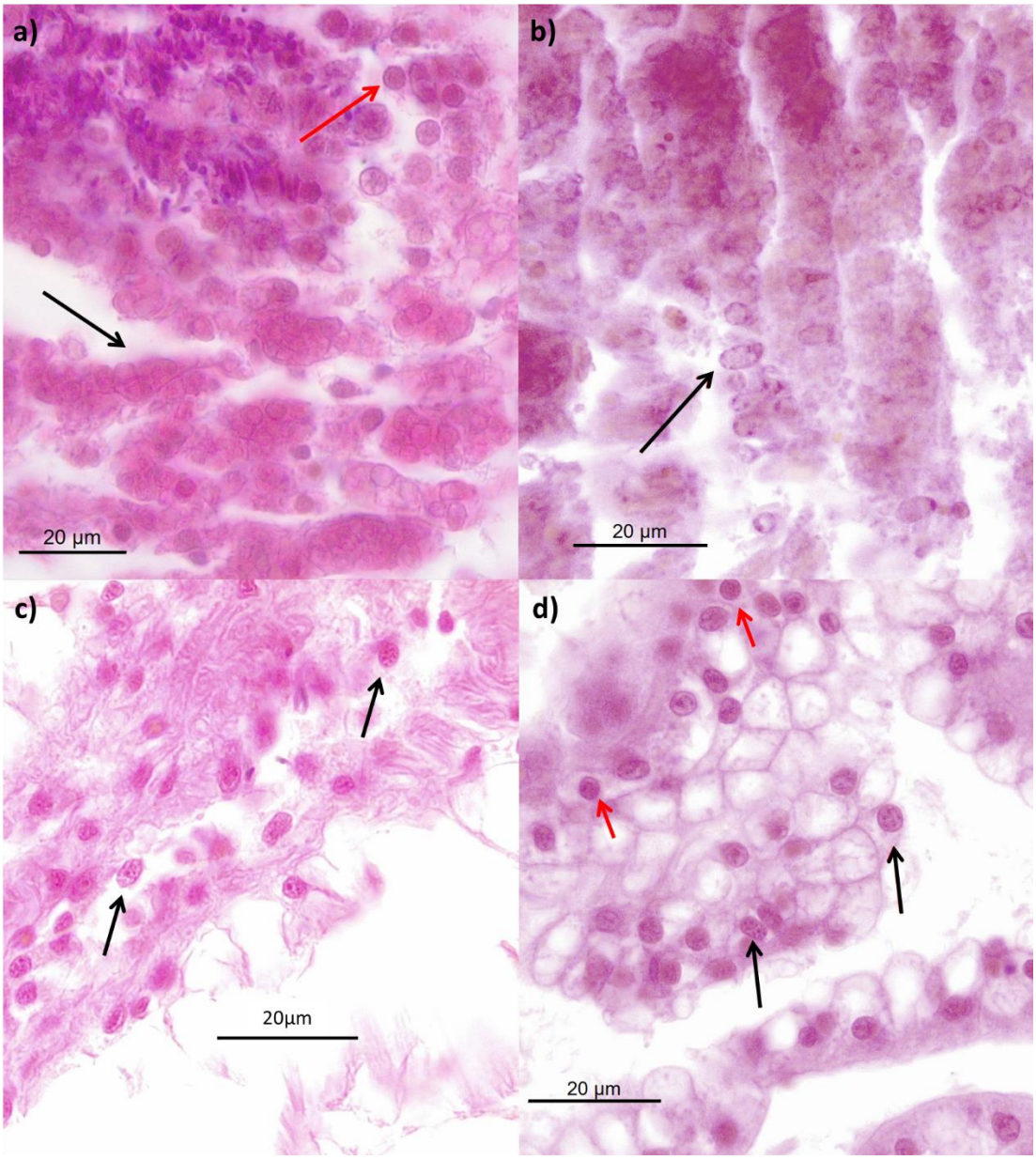


Figure 5: a) Multiple haplosporidia-like sporonts (black arrow) along with haemocytes (red arrow) in the connective tissue of *Cerastoderma edule*; b) a large number of haplosporidian spores (black arrow) in the mantle tissue of *C. edule*; c) multinucleate plasmodia (black arrow) in the connective tissue of *C. edule*; and d) multinucleate plasmodia (black arrow) along with haemocytes (red arrow) in the digestive gland of *C. edule*.

Ten histological sections of samples that were negative by PCR were screened and no evident signal of infection was found in those, and tissues, organs and sinuses presented good integrity and a clear structure.

4.4. Discussion

The present study describes for the first time, to the best of our knowledge, simultaneous infections with haplosporidians and *Vibrio* spp. in wild and fished *C. edule* populations along the Irish coast. Coinfections with two and, especially, three pathogens, were less frequent in this study compared to single infected individuals but were statistically consistent with the values expected by random chance, except for the coinfection with both *Minchinia* species which was overrepresented in our samples. Only three individuals presented a three-pathogen coinfection with *Minchinia* spp. and *Vibrio* spp., all located at Cork Harbour. Previous studies reported mortalities associated with coinfections of ostreid herpesvirus (OsHV-1 and variants) and *Vibrio* spp. in *C. gigas* in France (Petton et al., 2015; Azéma et al., 2016; Petton et al., 2019), as well as with the co-occurrence of different protozoa and bacteria in the noble pen shell *Pinna nobilis* in the Mediterranean Sea (Carella et al., 2020). Cork Harbour was the site with the oldest cockles, and 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). Nevertheless, older bivalves have also more time available to develop immunotolerance and defence mechanisms compared to their younger cohorts (Renault and Novoa, 2004; Coen and Bishop 2015). It may be the reason why cockles at Cork Harbour have not succumbed to the multiple infections. Moreover, methodological limitations may have masked multiple infections at a finer scale than the sampling can resolve. Consequently, future research should focus on the multiple infections at different scales, between sites and within the sites, in order to extend the understanding of the prevalence of coinfection and its distribution.

Even though Haplosporidia and *Vibrio* were found simultaneously in double infected individuals, no significant biological interaction was statistically identified between those pathogen groups. Unlike other studies that have reported that multiple pathogens can interact synergistically, with the presence of one pathogen enhancing the abundance and/or virulence of the other (De Lorgeril et al., 2018; Carella et al.,

2020). Concurrent infections by multiple pathogens species are more likely to occur in immune-depressed individuals, which are more vulnerable to multiple opportunistic infections (De Lorgeril et al., 2018; Carella et al., 2020). These findings highlight that there can be variation amongst pathogen groups and the primary pathogen-opportunistic pathogen interplay.

It is well known that environmental and ecological factors potentially influence mechanisms of disease transmission in marine systems, i.e., hydrodynamics, the biomass of infected animals, etc. (Petton et al., 2015). Similarities in the host environment, behaviour or susceptibility can cause correlations in the risk of infection between two parasites (Vaumourin et al., 2014). In this study, the proportion of double infected individuals with Haplosporidia and *Vibrio* was explained by risk factors (increased seawater temperature, reduced salinity and host condition) shared by the two pathogen groups, increasing the probability of infection by both infectious agents. Such confounding factors may influence host exposure and/or host susceptibility promoting simultaneous infections, even though involved pathogens do not interact biologically (Vaumourin et al., 2015). These factors may be naturally occurring or be outcomes of coastal development (e.g., pollution) or climate change (e.g., warming, increased precipitation events and subsequent freshwater loadings into bays and estuaries), and may impact the expression of diseases directly or indirectly (Coen and Bishop, 2015). The predicted warming, increased precipitation, and reduced salinity of coastal waters in temperate regions will provide new areas for the natural occurrence of pathogenic strains and may also impact the resilience of bivalve hosts (Rowley et al., 2014; Lynch et al., 2020a).

Consequently, spatial variability in the pathogen infection was detected driven by environmental and biological variables. The highest prevalence of coinfection with Haplosporidia and *Vibrio* spp. occurred in Dundalk Bay, which had a lower mean salinity throughout the year compared to the other sites. Gorrasi et al. (2020) observed that the abundance of *Vibrio* genus in water decreased along the salinity gradient within a marine saltern hypersaline environment. In general, marine vibrios are considered halotolerant, with no extreme halophiles reported within this genus (Gorrasi et al., 2020). Previous studies have highlighted the intolerance of haplosporidians to low salinity in *C. edule* (Albuixech-Martí et al., 2020), eastern oyster *Crassostrea virginica* (Ford and Haskin, 1982; Burreson and Ford, 2004) and

European flat oyster *Ostrea edulis* (Flannery et al., 2014). The high prevalence of haplosporidians at Dundalk Bay may be due to its commercial fishery activity. It is recognised that fishing and dredging may unbalance the interplay between infectious disease agents and their hosts in marine ecosystems (Rowley et al. 2014; Coen and Bishop, 2015). Cranfield et al. (1999, 2005) hypothesised that extensive dredging in Foveaux Strait of New Zealand, contributed to increase densities of Chilean oysters (*Ostrea chilensis*) and decrease densities of other filter-feeding organisms, that may have previously reduced the dispersal stages of the haplosporidian *Bonamia exitiosa*, enhancing disease transmission. The high cockle density found at Dundalk Bay may have facilitated the infection among the cockle population, due to the proximity of infected to non-infected cockles. The cultivated and harvested molluscs tend to have a much greater incidence of disease than wild molluscs, which may be because they are placed at high densities which enhance disease transmission and cause stress (competition for resources, space, etc.) among individuals (Ford, 2002).

Dungarvan Harbour also displayed a high prevalence of coinfection with Haplosporidia and *Vibrio* spp. compared to the other sites. Cockles from Dungarvan Harbour were the smallest and the youngest ones. Although it is accepted that older hosts have more time to accumulate disease-causing pathogens (Lafferty and Kuris, 2009; Breitburg et al., 2015), the susceptibility of small bivalves to viral and bacterial infections is generally greater than for larger adults (Renault and Novoa, 2004; Coen and Bishop 2015). Moreover, the proximity of an extensive *C. gigas* culture site at Dungarvan Harbour, where *Vibrio* spp. are endemic (pers. comm. with Marine Institute Ireland), can have promoted the presence of *Vibrio* in the environment. Particularly, *V. aestuarianus* and *V. splendidus*, which have been identified in this study, have been described as increasingly harmful pathogens of *C. gigas* aquaculture (Le Roux et al., 2002; Garnier et al., 2007; McCleary and Henshilwood, 2015). Caballes et al. (2012) demonstrated interspecific transmission of *Vibrio rotiferianus* between co-occurring echinoderm species.

Even though the natural host for OsHV-1 is *C. gigas*, this virus can behave opportunistically and infect other cohabiting bivalve species (Bookelaar et al., 2020). It has been previously reported in *C. edule*, in Ireland (Bookelaar et al., 2020) and in the Sydney cockle, *Anadara trapezia*, in Australia (Evans et al., 2017). Nevertheless, no presence of OsHV-1 µVar was detected in the study, despite the extensive farming

of *C. gigas* at Dungarvan Harbour, which has a history of OsHV-1-associated mortality events (Lynch et al., 2012; Prado-Alvarez et al., 2016). During summer 2018 the prevalence of OsHV-1 µVar in *C. gigas* at Dungarvan Harbour was confirmed by Kett et al. (unpublished data) to be 48.4% (n=92/190), with low mortality rates (max. of 6% at the high shore in July). Moreover, Bookelaar et al. (2020) observed OsHV-1µVar-infected *C. edule* (14.4%; n=36/250) and infected *C. gigas* (range of 0-27% per month; n=270) at Dungarvan Harbour from April 2015 to August 2015, with no recording of mortalities. Thus, the lack of OsHV-1 µVar detection in *C. edule* at Dungarvan Harbour in the current study may be because the virus remained within the oysters and was not shed from dying or dead oysters into the environment, as the high prevalence and low mortality recorded by Kett et al. (unpublished data) suggested. The smaller sample size (n=81) compared to the above studies, taken during spring and summer 2018 when the OsHV-1 µVar can have a major emergence due to the higher temperatures (Renault et al., 2014), may have also influenced the results.

Seasonal variability was also observed in this study, with the highest prevalence of coinfection with Haplosporidia and *Vibrio* spp. occurring in summer 2018, which coincided with the highest intensity of infection with *V. aestuarianus.* Additionally, lower occurrences of the haplosporidian and *Vibrio* species quantified were detected in the cooler spring 2019 compared to spring 2018 in this study. Burreson and Ford (2004) tested that low-temperature conditions caused a dramatic reduction in the prevalence of *Haplosporidium nelsoni* in *C. virginica*. Conversely, a higher prevalence of *Bonamia ostreae* in European flat oyster *O. 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. It is also well-known that bacteria belonging to the genus *Vibrio* are strongly thermo-dependent and their occurrence is expected to rise with increasing temperature conditions (Garnier et al., 2007; Cotter et al., 2010; Vezzulli et al., 2010). Most of the reported *Vibrio*-associated mass mortalities in marine invertebrates have occurred during the summer when seawater temperatures were higher than 18°C (Le Roux et al., 2002; Garnier et al., 2007; Vezzulli et al., 2010). Therefore, high temperatures in summer might have triggered a major pathogen propagation and, consequently, a higher prevalence of two-pathogen coinfection. While lower temperatures in winter, with the lowest prevalence of two-pathogen

coinfection, would have kept the pathogens at a sub-lethal level in the hosts reducing the transmission between cockles. It has been observed that *Vibrio* spp. may enter into a dormant state when the environmental conditions become unfavourable (i.e. suboptimal or reduced temperature, elevated salinity, low nutrient concentration and extreme pH) (Lin and Schwarz, 2003; Nowakowska and Oliver, 2013; Neogi et al., 2018). The low number of coinfected individuals in winter might also suggest that most *C. edule* had succumbed to infection in autumn after the peak in summer.

It was also observed that with the higher temperatures in summer and spring the presence of epiflora on the cockle shells was more frequent, which may have consequences on cockle susceptibility to infectious agents. As seen in shellfish aquaculture, the presence of biofouling on the stock can have a direct impact, causing physical damage, biological competition, mechanical interference and environmental modification, while infrastructure is also impacted (Fitridge et al., 2012). Microalgae have been pointed to as a repetitive and substantial source of bacterial inoculation into the bivalve larval culture systems (Salvesen et al., 2000). Microalgae may promote and/or inhibit bacterial growth by the production of organic exudates and toxic metabolites (Salvesen et al., 2000). For instance, *Vibrio* species such as *V. splendidus* or *V. parahaemolyticus* have been found to be associated with microalgae (Kumazawa et al., 1991; Rehnstam-Holm et al., 2010; Notaro et al., 2021). Therefore, the presence of epiflora on the shells might have also exposed the cockles to pathogens, in particular *Vibrio* species.

Both species of *Minchinia, M. tapetis* and *M. mercenariae*-like, were found to occur together significantly more often than expected by random chance, indicating a positive two-way association between both species. However, previous studies examining pairwise coinfections indicate that antagonistic interactions between similar types of parasites may be common (Dobson, 1985; Johnson and Hoverman, 2012). A possible explanation of the established synergetic interaction is that, despite both *Minchinia* species being closely related, they exhibit differences in their pathogenic effect and use of resources within the host. Carballal et al. (2020) observed that the distribution of infection with *M. tapetis* and *M. mercenariae*-like haplosporidian in cockles *C. edule* in Galicia was different throughout the cockle tissues. *M. mercenariae*-like haplosporidian was observed in the connective tissue of the digestive gland, gills and gonad, while *M. tapetis* was only observed in the

digestive gland (Carballal et al., 2020). Moreover, shared susceptibility factors and shared environmental co-factors not captured in the models may have also influenced this synergetic association. Further research is needed to reveal the underlying mechanism of this synergetic association in *C. edule* and the role that host exposure and host habitat play in it. Given the heterogeneous and fluctuating environment of intertidal habitats, the study of the microscale variation within the cockle beds may also shed light on the ultra-local environmental and biological factors affecting the prevalence of double infected individuals with *Minchinia* and the positive association detected.

In brief, single infections with Haplosporidia and *Vibrio* were more frequent than coinfected individuals, as expected by random chance. The proportion of double infected individuals with haplosporidian and *Vibrio* was driven by environmental and biological factors, triggering a spatial and temporal variability in the distribution of coinfection. The higher temperature in warmer months and presence of epiflora, which can be associated with pathogens, on the cockle shells may have promoted the risk of coinfection, along with low salinity and the host condition in some of the sample sites. The close proximity to other infected host species may have also influenced the coinfection distribution in *C. edule*. Similarly, fished *C. edule* populations with high cockle densities might have been more exposed to the infection. Therefore, those factors may conform the ideal scenario for simultaneous infections, although the pathogen groups showed no biological interaction between them. Nevertheless, individuals coinfected with both *Minchinia* species were observed significantly more often than expected by random chance. Likewise, a positive two-way association between both *Minchinia* species was statistically confirmed, which may be due to their different pathogenic effects and use of resources within the host, although further research on the underlying mechanism of this association is needed. Our findings shed light on the complex interactions between multiple aetiological agents associated with host diseases in the frame of a natural system. This study highlights the consequences of the coinfections are not simply the sum of the effects caused by each infectious agent, but the result of a complex combination of direct and indirect pathogens effects, the host ecology, and the environmental context.

These results are framed into the current scenario of climate change, which involves oceans warming on average by 0.6°C in the next 100 years (Wiltshire et al.,

2008), with European seas especially affected (Philippart et al., 2011). The sea surface temperature around the United Kingdom and Ireland has increased at six times the rate of the global average, particularly jeopardizing species that have small thermal tolerance windows (Frost et al., 2012). As a result, previously appropriate habitats for cockles may no longer be suitable due to changes in thermal regime, oxygen levels or altered rainfall patterns (Morgan et al., 2013; Coen and Bishop, 2015). Climate change may also be expected to have a large effect on disease occurrence, especially in host species showing strong relationships between temperature/salinity and diseases, as seen for the cockle populations studied, and for many other molluscan host-parasite systems (Coen and Bishop, 2015; Allam and Raftos, 2015; Lynch et al., 2020a). The physiological stress may also cause the host to become immune-compromised and unable to suppress an infection (Suttle, 2007). Therefore, climate change could be the driver of the spreading and range expansions of infectious agents, such as bacteria, haplosporidians and viruses, in the marine ecosystem and may lead to more frequent pathogen outbreaks (Burreson and Ford, 2004; Suttle, 2007; Rowley et al., 2014; Coen and Bishop, 2015). As global climate change and anthropogenic impacts continue to modify the distribution and abundance of hosts and parasites and the ways in which they interact, integral studies like ours are important since concepts gained might be applied to other associations and to a changing environment.

A final important point to emphasise is that the relative potential of coinfections to cause the emergence of zoonoses in wild animals remains to be fully assessed (Hoarau et al., 2020). Given that two-thirds of emerging infectious diseases are zoonoses, with nearly 70% originating from wildlife (Hoarau et al., 2020), better knowledge of the interactions of infectious agents in wild reservoirs, such as cockles, will provide key insight for the understanding and management of spillover processes. Although challenging, findings from this study highlight the necessity to integrate data pertaining to host ecology, pathogen diversity, biogeography and seasonality of the disease and the environmental influence. This approach should be consistently applied to provide a more detailed picture of coinfection dynamics in individual hosts and communities over time.

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CHAPTER 5

Connectivity dynamics in Irish mudflats between microorganisms including *Vibrio* spp., common cockles *Cerastoderma edule*, and shorebirds

Connectivity dynamics in Irish mudflats between microorganisms including *Vibrio* spp., common cockles *Cerastoderma edule*, and shorebirds

This chapter is published as follows:

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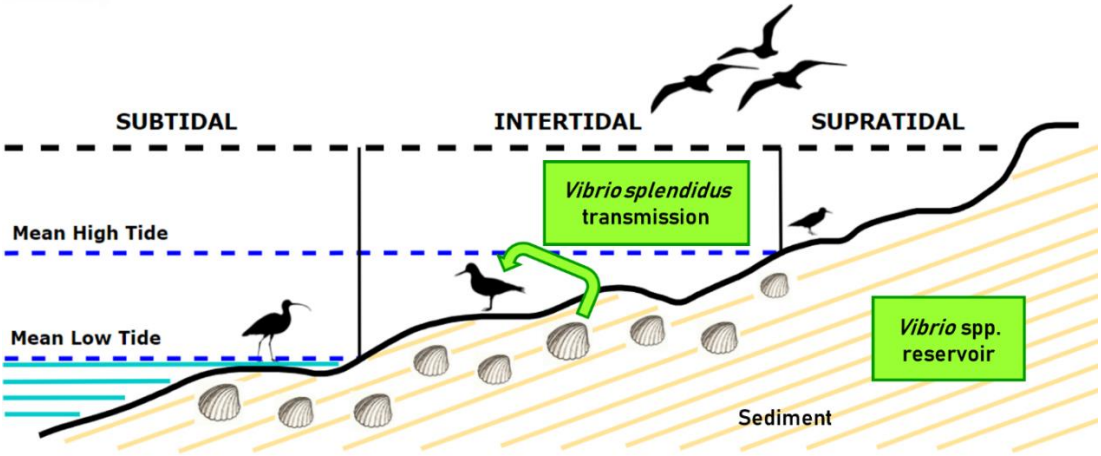
Abstract

Shellfish, including the key species the common cockle *Cerastoderma edule,* living and feeding in waters contaminated by infectious agents can accumulate them within their tissues. It is unknown if microbial pathogens and microparasites can subsequently be transmitted via concomitant predation to their consumers, including shorebirds. The objective of this study was to assess if pathogens associated with *C. edule* could be detected seasonally in the faeces of shorebirds that feed on *C. edule* and in the physical environment (sediment) in which *C. edule* reside, along the Irish and Celtic Seas. Two potentially pathogenic global groups, *Vibrio* and Haplosporidia, were detected in *C. edule*. Although Haplosporidia were not detected in the bird faeces nor in the sediment, identical strains of *Vibrio splendidus* were detected in *C. edule* and bird faecal samples at sites where the oystercatcher *Haematopus ostralegus* and other waders were observed to be feeding on cockles. *Vibrio* spp. prevalence was seasonal and increased in *C. edule* and bird faecal samples during the warmer months, possibly due to higher seawater temperatures that promote the replication of this bacteria. The sediment samples showed an overall higher prevalence of *Vibrio* spp. than the bird faecal and *C. edule* samples, and its detection remained consistently high through the sites and throughout the seasons, which further supports the role of the sediment as a *Vibrio* reservoir. Our findings shed light on the fact that not all pathogen

groups are transmitted from prey to predator via feeding but bacteria such as *V. splendidus* can be. As most of the wading birds observed in this study are migratory, the results also indicate the potential for this bacterium to be dispersed over greater geographic distances, which will have consequences for areas where it may be introduced.

Keywords: *Cerastoderma edule*, shorebirds, sediment, *Vibrio*, Haplosporidia, trophic transmission, disease connectivity.

Graphical abstract:



Highlights:

- Haplosporidia transmission to shorebirds was not detected.
- Transmission of *Vibrio splendidus* to shorebirds, via *Cerastoderma edule* consumption.
- Role of sediment as a *Vibrio* reservoir, where the bacteria can find a favourable environment for overwintering.

5.1. Introduction

Mudflats are coastal wetlands that form in sheltered intertidal areas such as bays and estuaries, where sediments have been deposited by tides or rivers. Mudflats support a large population of wildlife and are a key habitat for many migratory shorebirds, as well as for certain species of molluscs, crabs, and fish (Thieltges et al., 2018). The common cockle *Cerastoderma edule* is widely distributed in the Atlantic and is one of the main non-cultured bivalve species harvested in western European bays and estuaries, where population densities of 10,000 per m^2 have been recorded (Tyler-Walters, 2007), making them a useful model species for this study. Furthermore, common cockles play a key role as an ecosystem engineer, controlling or influencing processes such as bioturbation and water filtration, increasing the productivity of sedimentary habitats, which underpin marine food webs and biodiversity (Malham et al., 2012; Magalhães et al., 2017; Carss et al., 2020). In the marine food chain, *C. edule* is an important food source and is a link between primary producers (phytoplankton, phytobenthos) and consumers such as crabs, shrimps, fish and birds (Magalhães et al., 2017). Cockles, inhabiting the intertidal, are easily available to a variety of predators since they are found only a few millimetres beneath the sediment surface, they may even emerge at the surface when abundance is high, because of sediment erosion, or when they are stressed, such as by oxygen deficiency or trematode infection (Lassalle et al., 2007). One of the main consumers feeding on up to 300 cockles per day is marine bird populations (Malham et al., 2012), whose feeding ecology may shape their diverse and multiple endoparasitic fauna (Hoberg, 1996), responsible for significant effects on marine birds (Muzaffar and Jones, 2004). Trophically transmitted parasites and pathogens are central elements in most aquatic food webs, given that foraging behaviour and diet facilitate the transmission of a variety of pathogens and endoparasites through marine invertebrate and vertebrate species (Lafferty et al., 2006; Lafferty et al., 2008). Concomitant predation is the most common way for parasites and pathogens to become prey, which occurs when an infected host is eaten by a predator. Because of the high productivity of aetiological agents, their unique nutritional composition and their pathogenicity in hosts, their consumption affects food web structure, energy transfer and disease dispersals (Johnson et al., 2010).

Although parasitism contributes significantly to the biodiversity of mudflat ecosystems, the composition of parasites in the food web of the mudflat ecosystems has been understudied (Thieltges et al., 2018). Cockles inhabiting those habitats can accumulate agents that are potentially pathogenic (Zannella et al., 2017) and act as a reservoir for subsequent infection of other species (Carss et al., 2020). Cockles are hosts to a diverse range of macroparasites (Fermer et al., 2011), but may also be infected by a range of pathogens including protists, bacteria and viruses (Longshaw and Malham, 2013). In turn, Newman et al. (2007) confirmed that infectious diseases are an important cause of mortality events and individual bird deaths, especially among coastal and freshwater aquatic birds, pointing out bacterial infections as a frequent cause of mass mortality events.

A wide range of bacterial infections occurs in free-ranging birds (Muzaffar and Jones, 2004), with many copiotrophic bacteria, such as *Vibrio* spp., widely distributed in estuarine and coastal ecosystems (Vezzulli et al., 2016; Jesser and Noble, 2018). A large number of *Vibrio* species are associated with marine organisms like fish, molluscs and crustaceans, in commensal or pathogenic relations (Romalde et al., 2014). Some species such as *Vibrio aestuarianus*, *Vibrio splendidus* or *Vibrio tapetis* have been associated with mortalities of different molluscan species, seriously affecting their culture and causing high losses in hatcheries as well as in natural beds (Allam et al., 2002; Waechter et al., 2002; Gay et al., 2004; Paillard et al., 2004; Prado et al., 2005; Garnier et al., 2008). For other species, ecological importance has been demonstrated, such as *Vibrio crassostreae, Vibrio breoganii,* and *Vibrio celticus*, which form part of the molluscan microbiota (Romalde et al., 2014), or *V. mediterranei,* found in the microbiota of marine fish (Egerton et al., 2018). In addition, *Vibrio* bacteria can persist in high abundance and/or can be found during cold and unfavourable environmental conditions in the sediment compartment (Vezzulli et al., 2013; Vezzulli et al., 2015). Azandégbé et al. (2010) reported the occurrence of *V. aestuarianus* in sediment at two *C. gigas* farms in France and suggested that this bacterium might subsist during the cold seasons in the sediment and rise again under favourable conditions.

The haplosporidian protists are also widespread and have been associated with some of the most serious epizootic mortalities of commercially important bivalves, including cockle population crashes in The Netherlands and UK (Burreson and Ford,

2004; Engelsma et al., 2011; Longshaw and Malham, 2013; Arzul and Carnegie, 2015; Carnegie et al., 2016). A number of studies have reported the detection of *Minchinia* (Engelsma et al., 2011; Longshaw and Malham, 2013; Ramilo et al., 2018; Albuixech-Martí et al., 2020; Lynch et al., 2020) and *Haplosporidium* in cockles (Azevedo et al., 2003; Engelsma et al., 2011), and *Urosporidium* parasitizing trematodes or turbellaria infecting cockles (Carballal et al., 2005). Cockles are a known food source for shorebirds; however, to the best of our knowledge, Haplosporidia have not been previously associated with birds.

Viral diseases play an increasingly important role in the health of aquatic birds, as has been suggested in previous studies (Daoust et al., 1998; Converse and Kidd, 2001; Friend et al., 2001). Particularly, herpesviruses have been previously associated with respiratory and enteric disease and mortality among seabirds and waterfowl (Hubálek, 2004; Quesada et al., 2011; Niemeyer et al., 2017). In turn, even though the natural hosts for ostreid herpesvirus type-1 (OsHV-1) are oysters, Bookelaar et al. (2020) recently determined the potential for *C. edule* to become infected with and to act as an alternate host of OsHV-1 µVar. The presence of herpesviruses in the sediment has been also described. Honjo et al. (2012) reported for the first time Cyprinid herpesvirus 3 (CyHV-3) DNA in sediments of natural lakes and ponds suggesting that sediment could act as a reservoir for CyHV-3 in natural freshwater environments. Subsequently, Evans et al. (2017) detected OsHV-1 in the mangrove sediments from an estuary in Australia.

Therefore, the environmental reservoirs along with the hosts may largely influence the ecology of those pathogen groups by favouring their survival and dispersion in the environment and also serving as a vector for their associated disease. Free-living birds might be involved in the carriage of microbial pathogens not only as biological carriers (the pathogen multiplies in the avian body) but also as mechanical carriers (the pathogen does not multiply in or on the bird) (Hubálek, 2004). The pathogen can be located on the surface of the bird's body or pass through the digestive tract, being viable when excreted (Hubálek, 2004). Likewise, birds are hosts for many parasites that sometimes serve as vectors of infectious agents (Hubálek, 2004). Birds, including waterfowl, can be hyperparasitized by microsporidians that infect their parasites (Slodkowicz-Kowalska et al., 2006; Malcekova et al., 2013). Hyperparasitism by microsporidia might influence trophic relationships between

invertebrate hosts such as *C. edule* and vertebrate hosts such as birds, releasing the secondary host from metacercarial infections (Fermer et al., 2009). Therefore, birds may play an important role as a reservoir of infection and/or incidental carriers influencing pathogen transmission, dispersal, and disease dissemination. The potential for transport and dissemination of certain pathogenic microorganisms by free-living birds is of concern and is the subject of increased vigilance. For instance, the roles of wild birds in the spread of avian flu and tick-borne viruses are well recognised (Yun et al., 2015; Xu et al., 2016).

Parasite-inclusive food web studies have so far mainly considered macroparasites; however, the large diversity of microparasites and pathogens in mudflat ecosystems remains to be fully explored (Thieltges et al., 2018). Even though it is well-known that infectious agents may incur costs for their avian hosts at an individual level (Muzaffar and Jones, 2004), the trophic transmission of microbial and parasitic infections in aquatic birds and their influence on disease dynamics and dissemination are difficult to establish. Further complications arise from the fact that some host groups such as birds are often transient components of the local food web that can carry parasites and pathogens acquired elsewhere (Thieltges et al., 2018). Polymerase chain (PCR)-based techniques for detecting prey remains in the gut, faeces and regurgitates of predators can be applied to study complex trophic interactions in the field; however, their application is still a challenge. The lack of sensitivity, short post-ingestion detection periods and cross-amplification problems are the major difficulties in detecting degraded, semi-digested DNA (King et al., 2008). Nevertheless, following stringent contamination control strategies when collecting samples, prompt preservation of those samples and assay optimization can prevent these problems (Harper et al., 2005; Martin et al., 2006; Read et al., 2006; Harwood et al., 2007).

The presence of the *Vibrio* genus in *C. edule* has been confirmed on the eastern and southern Irish coasts (Albuixech-Martí et al., 2021). Likewise, there have been previous records of infected *C. edule* populations with haplosporidian protists such as the genus *Minchinia* (Albuixech-Martí et al., 2020; Lynch et al., 2020), or with ostreid herpesvirus type-1 (OsHV-1) (Bookelaar et al., 2020), as well as records of microsporidian species parasitizing digeneans that infect cockle populations in those areas (Fermer et al., 2009; Fermer et al., 2011). Therefore, the objective of this study

was to assess if pathogens associated with *C. edule* (*Vibrio* spp., Haplosporidia spp., ostreid herpesvirus-1 microVar, Microsporidia spp.) could be detected seasonally in the sediment and the faeces of shorebird species at sample sites along the Irish and Celtic Seas. This study used PCR-based techniques adopting strict contamination control strategies and optimisation of the storage, extraction, and purification of the DNA from bird faecal droppings, sediment, and cockles. Shorebird species present were recorded while foraging, with particular attention to cockle consumption. The observational data from shorebirds was assessed in relation to *C. edule* DNA presence and pathogen detection in faecal samples. The spatial and seasonal influence in the detection of the infectious agents in cockles, shorebird faecal samples as well as sediment was also evaluated. Findings will provide an insight into the pathogen community present in *C. edule*, shorebird populations and sediment, establishing the potential of the different pathogen groups to persist in these three natural compartments and be trophically transmitted from *C. edule* to shorebirds. This study highlights the role of the shorebirds as carriers of infectious agents as well as the role of the sediment in the pathogen persistence.

5.2. Materials and Methods

5.2.1. Sample sites

Cockles, faecal samples and sediment samples were collected seasonally from April 2018 to April 2019 at Cork Harbour (Ringaskiddy and Cuskinny) on the south coast (Celtic Sea) of Ireland, and from July 2018 to April 2019 at Youghal Bay and Dungarvan Harbour, the latter the site of extensive farming of Pacific oysters (*Crassostrea gigas*), on the south coast (Celtic Sea), as well as at the commercial fishery at Dundalk Bay (Annagassan and Cooley) on the northeast coast (Irish Sea) (Figure 1). The six locations are bays with intertidal sand and mudflats, where a variety of bivalve species inhabit. They 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). Moreover, Cuskinny is classified as Marsh Nature Reserve and Dundalk Bay is a classified Bivalve Mollusc Production Area and it has supported a

commercial dredge cockle (*Cerastoderma edule*) fishery since 2001 (Hervas et al., 2008).

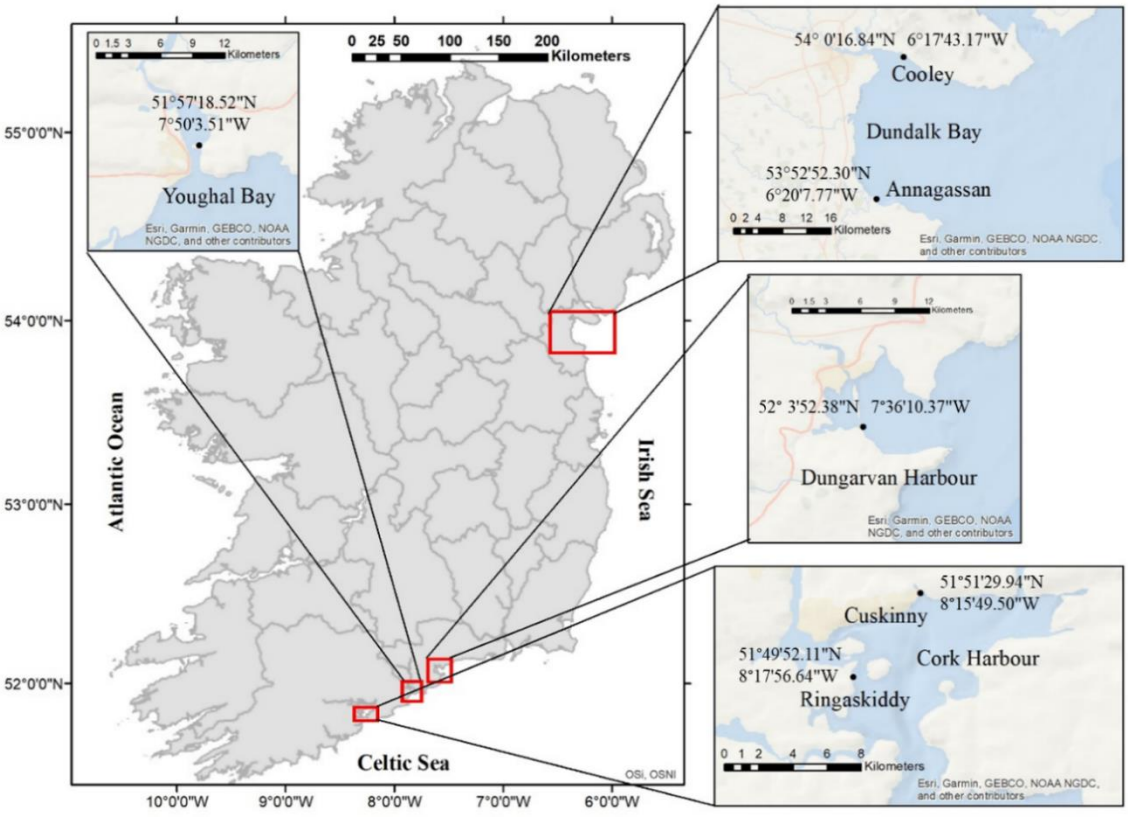


Figure 1: Map of Ireland highlighting the sample sites with coordinates (ArcGIS Desktop 10.5.1; www.esri.com)

Although the cockle densities *in situ* were not measured, observations of cockle abundances during the samplings were in accordance with the reference densities previously recorded in those sites. *C. edule* densities in the selected sites varied from higher densities, 9.33 ± 3.5 ind/m^2, in Dundalk Bay (Shellfish Stocks and Fisheries Review, Marine Institute and BIM, 2017) to lower densities in sites that are not fished: 1.6 ± 2.1 ind/m^2 in Youghal Bay; 0.8 ± 1.7 ind/m^2 in Cork Harbour; and 0.4 ± 1.3 ind/m^2 in Dungarvan Harbour (Fermer et al., 2011).

5.2.2. Bird observations

Video recording of shorebirds while foraging at low tide in the study areas was carried out seasonally from April 2018 to April 2019, following the specifications of Martins et al. (2013). Recordings were carried out with a digital camcorder (CX405B,

Sony) with a 30x optical zoom. The video recording was done prior to sampling to avoid disturbance. The average duration of the films was 5 to 10 minutes, each one taken from a different perspective of the bay while filming the main bird aggrupations. Therefore, it is highly unlikely that pseudoreplication (i.e., filming the same individual more than once) affected our dataset and conclusions to any significant extent. The recording was done in the aerially exposed cockle bed, where the faecal samples and the sediment samples were collected at low tide. The viewing of the videos was carried out to list the bird species, their number and behaviour i.e., foraging and feeding or not, at the time of the filming. Following the descriptions in Lewis and Tierney (2014), shorebird behaviour was classified into two categories: (1) foraging/feeding: the active or passive search of food and feeding; and (2) other: this category was used for all other behaviours that do not assign to foraging, including roosting, standing, preening, loafing, swimming and others. Prey identity was not examined. However, in some cases, analysis of video recordings allowed the distinction of the consumption of bivalves, even though identification of specific species could not be done even with the best quality recordings.

5.2.3. Sample types

Cockles were collected by hand raking from the intertidal area of each site at spring tide (0.5m-1m) and seasonal intervals. The sample size, outlined in Table 1, was dependant on the available abundance at each site and season. Cockles were kept at ambient temperature during the fieldwork and were processed either on the same day or were held overnight at 4°C and were processed the next day.

Sediment and seabird faecal samples were collected as near as possible to the cockle collection area (Table 1). As food retention time in the digestive tracts of birds is short - for instance, for red knots (*Calidris canutus*) it is 20–50 min (Piersma et al., 1993) -, the collection of samples was done after 30 min from the start of the video recording to ensure that the bird faeces collected were composed of food remains found and digested by the shorebirds feeding on prey from that foraging site (Martins et al., 2013). The collection of samples was conducted following stringent contamination control strategies, such as using sterilised material and avoiding taking any substrate in contact with the faecal matter. One sterilised swab was used to collect a small amount (around $5mm^3$) of the bird faecal waste and a second swab was used

to collect the same amount of sediment nearby to the stool. Each sample was transferred into an individually labelled 2ml sterile Eppendorf tube.

Wherever possible, faeces were collected fresh to minimize further enzymatic action and, consequently, degradation of the DNA, although freshness is not always easy to determine in the field (King et al., 2008). Preservation of faecal samples has mainly been by freezing in a range from -20 °C to -80 °C (Harper et al., 2005; Martin et al., 2006; Read et al., 2006; Harwood et al., 2007). Seabird faeces and the sediment samples were stored in an insulated bag with iced packs during the fieldwork and, once in the lab, they were frozen at -80°C until their processing to accelerate freezing and halt DNA-destroying enzymatic processes.

Table 1: Number of collected cockles (C.), bird faecal samples (F.) and sediment samples (S.) by site and season (Sp.18: spring 2018; Sum.18: summer 2018; Aut.18: autumn 2018; Wint.18/19: winter 2018/19; Sp.19: spring 2019).

Dates	**Cork Harbour - Ringaskiddy**			**Cork Harbour - Cuskinny**			**Youghal Bay**			**Dungarvan Harbour**			**Dundalk Bay – Annagassan**			**Dundalk Bay -Cooley**		
	C.	***F.***	***S.***	***C.***	***F.***	***S.***	***C.***	***F.***	***S.***	***C.***	***F.***	***S.***	***C.***	***F.***	***S.***	***C.***	***F.***	***S.***
Sp. 18	29	4	4	31	2	2	-	-	-	-	-	-	-	-	-	-	-	-
Sum. 18	12	-	-	27	-	-	11	4	4	51	3	3	43	6	6	17	10	10
Aut. 18	31	10	10	29	10	10	16	10	10	58	10	10	30	10	10	30	10	10
Win. 18/19	15	10	10	23	10	10	30	10	10	30	10	10	30	10	10	30	10	10
Sp. 19	30	10	10	30	10	10	12	10	10	30	5	5	30	10	10	30	10	10
Totals	**117**	**34**	**34**	**140**	**32**	**32**	**69**	**34**	**34**	**169**	**28**	**28**	**133**	**36**	**36**	**107**	**40**	**40**

(-) No samples collected

5.2.4. Sample processing and diagnostic methods

A small piece of gill tissue (2-5mm^2 of tissue) from each cockle was taken and stored at -20°C for molecular assays. Genomic DNA from the cockle gill tissue was extracted using the chelex-100 extraction method (Walsh et al., 1991; Lynch et al., 2008). The DNA of seabird faecal samples, as well as the sediment samples, were extracted by Qiagen QIAamp DNA Stool Kit, following the protocol of Zeale et al. (2011), with modifications from Dr James Nicholls (www.protocols.io/view/dna-extraction-from-avian-faeces-stored-in-ethanol-ve6e3he) and Sarah Davies (*pers*

comm). Negative controls during DNA extraction were included to screen for potential contamination by prey DNA between samples and provide a higher degree of confidence in the assay protocol. Once extracted, the samples were used to determine the European cockle species (*Cerastoderma edule* (Linnaeus, 1758) or *Cerastoderma glaucum* (Poiret, 1789)) present and for subsequent pathological screening.

The quantity of target DNA remaining after storage, extraction and purification of the DNA, affects the successful amplification of that DNA from faecal material (King et al., 2008). It should be highlighted that faecal DNA is often degraded, the risk of contamination is high, and PCR inhibitors may likely be co-extracted (King et al., 2008). Therefore, the quantity and the quality of the extracted DNA from seabird faeces and sediment samples were established using NanoDrop 1000 Spectrophotometer to avoid the diagnosis of false negatives due to low DNA quantity and/or DNA of poor quality. Likewise, the quantity and the quality of the extracted DNA from a subsample (n=120) of the collected cockles were also tested. DNA purity was assessed by determining the ratio of spectrophotometric absorbance of each extracted sample at 260/280 nm (A260/A280 ratio; an indicator of protein or phenol contamination). Pure DNA extraction should have an A260/A280 ratio ~1.8, higher values indicate the presence of RNA along with DNA.

Polymerase chain reaction (PCR) was carried out in the cockle, seabird faecal and sediment samples to amplify the nuclear DNA markers ITS-for / ITS Ce-R / ITS Cg-R to differentiate between the presence of *C. edule*, *C. glaucum* species or hybrids (Freire et al., 2011; Table 2). Purified DNA of *C. edule* was used as a positive control. Two positive controls were included in each PCR assay to confirm that the amplification worked. Likewise, negative controls (two replicates with ddH_2O) were included in each PCR assay to confirm the absence of contamination. Amplification was conducted following the reaction mixture and thermocycling conditions, as well as the visualisation of the product, described in Albuixech-Martí et al. (2020).

Standard PCR screening in cockle, seabird faecal and sediment samples was conducted using generic primers Vib1F/2R (Thompson et al. 2004; Table 2) following the protocol modified by Vezzulli et al. (2012) (optimised by Davies, C. E.) for the detection of *Vibrio* genus. A total of 1µl of DNA per individual was used in a total volume of 25µl of the reaction mixture containing: 12.5µl of Taq 2X Master Mix,

0.125µl of forward and reverse primers (100mM) and 11.25µl of ddH_2O. Cycling conditions consisted of an initial denaturation of the sample at 94°C for 10 min followed by 30 cycles of 94°C for 30s, 58°C for 30s, 72°C for 60s and a final elongation at 72°C for 10 min. Purified DNA of the pathogen was used as a positive control. Two positive controls were included in each PCR assay to confirm that the amplification worked. Likewise, negative controls (two replicates with ddH_2O) were included in each PCR assay to confirm the absence of contamination. Electrophoresis of the amplification products was conducted in a 2% agarose gel. The expected amplified product (amplicon) size for *Vibrio* spp. was 120bp.

Standard PCR screening for haplosporidian detection was carried out in the cockle samples (n=735), likewise in a subsample of seabird faecal and sediment samples (n=168), using generic haplosporidian HAP-F1 and HAP-R3 primers that amplify small regions of the SSU rDNA of most haplosporidian parasites (Renault et al., 2000; Molloy et al., 2012; Table 2). Purified haplosporidian DNA was used as a positive control. Two positive controls were included in each PCR assay to confirm that the amplification worked. Likewise, negative controls (two replicates with ddH_2O) were included in each PCR assay to confirm the absence of contamination. Amplification was conducted following the reaction mixture and thermocycling conditions, as well as the visualisation of the product, described in Albuixech-Martí et al. (2020).

Standard PCR screening for *Minchinia* spp. detection was carried out in the cockle samples that were positive for Haplosporidia by conventional PCR (n=277), likewise in a subsample of seabird faecal and sediment samples (n=168), using specific primers for *Minchinia tapetis* (TAP-For/Rev) and *Minchinia mercenariae*-like (MER-For/Rev) (Albuixech-Martí et al., 2020; Table 2) in the appropriate pairings and in separate PCR reactions. Two positive controls (purified DNA of the respective *Minchinia* species) were included in each PCR assay to confirm that the amplification worked. Likewise, negative controls (two replicates with ddH_2O) were included in each PCR assay to confirm the absence of contamination. Amplification was conducted following the reaction mixture and thermocycling conditions, as well as the visualisation of the product, described in Albuixech-Martí et al. (2020).

Standard PCR screening for ostreid herpesvirus type-1 (OsHV-1) and variants was carried out in cockle samples (n = 735), likewise in a subsample of seabird faecal and sediment samples (n=226), using specific primers OHVA/OHVB that amplify small products of the ORF4 gene in the OsHV-1 virus and its variants (Lynch et al., 2013; Table 2). Purified DNA of the pathogen was used as a positive control. Two positive controls were included in each PCR assay to confirm that the amplification worked. Likewise, negative controls (two replicates with ddH_2O) were included in each PCR assay to confirm the absence of contamination. Amplification was conducted following the reaction mixture and thermocycling conditions described in Lynch et al. (2013). Electrophoresis of the amplification products was conducted in a 2% agarose gel. The expected amplified product (amplicon) size for the OsHV-1 virus and its variants was 385bp.

Standard PCR using specific primers (MicIF1-MicIR1 and MicIF2-MicIR2, Table 2) (Lynch, unpublished data confirmed to be specific to Microsporidia spp. by Sanger sequencing) to detect two unidentified microsporidian species was carried out in cockle samples (n = 735), likewise in a subsample of seabird faecal and sediment samples (n=116). Purified microsporidian DNA was used as a positive control. Two positive controls were included in each PCR assay to confirm that the amplification worked. Likewise, negative controls (two replicates with ddH_2O) were included in each PCR assay to confirm the absence of contamination. Both reaction mixture and thermocycling conditions were the same as used with the PCR for OsHV-1 and variants, as well as the visualisation of the product. The expected amplified product (amplicon) size for microsporidian species was 180bp.

Quantitative PCR (qPCR) for detection and quantification of *Vibrio aestuarianus* in the cockle, seabird faecal and sediment samples that were positive for generic *Vibrio* screening was performed using specific primers for detection of the molecular chaperone *dnaJ* gene, following the protocol of McCleary and Henshilwood (2015) (Table 3). Negative template controls consisting of 5 µl of double-distilled water (ddH_2O) were included in triplicates in each run. 5 µl of *V. aestuarianus* DNA suspension was used in triplicates as positive controls in each run. All samples were performed in triplicates using 5 µl of DNA. C_t (Cycle threshold) values were used to determine PCR quantification and detection limits by Thermo Hybaid PCR express thermal cycler. A tested sample was considered positive if its

mean C_t value was below 37. Six prepared 1:10 serially diluted *V. aestuarianus* DNA standards were tested in triplicates as stated in McCleary and Henshilwood (2015). Standard curves aim at checking the efficacy of the PCR reaction and at estimating the number of bacterial DNA copies present in tested samples. Results were interpreted by linear regression for slope analysis, and the PCR amplification efficiency (E) was calculated according to: $E = [10^{1/(-slope)}] - 1$ (McCleary and Henshilwood, 2015). Absolute quantitation for DNA copies was carried out by comparing the C_t obtained against the standard curve with known copy numbers.

Table 2: Description of PCR primer pairs showing sequences for each forward and reverse primer and expected product size.

	Primer sequence (5'–3')			
Primer pair	**Forward**	**Reverse**	**Product size (bp)**	**Primer specificity**
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*
Vib1-F Vib2-R	GGC GTA AAG CGC ATG CAG GT	GAA ATT CTA CCC CCC TCT ACA G	120	*Vibrio* spp.
HAP-F1 HAP-R3	GTT CTT TCW TGA TTC TAT GMA	AKR HRT TCC TWG TTC AAG AYG A	350	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
OHVA OHVB	TGC TGG CTG ATG TGA TGG CTT TGG	GGA TAT GGA GCT GCG GCG CT	385	Ostreid herpesvirus type-1 and variants
MicIF1 MicIR1	GTG GAC GCT AGT CTC ACA GGT T	TTG CAC CAG AAG GTT TAC GAC ACA T	180	Microsporidia sp.1
MicIF2 MicIR2	ATG CAT GCG TAA GCG AAG CAG TTA T	TCT CTT GCA CCA GAA GGT TTA CGA C	180	Microsporidia sp.2

Table 3: Description of qPCR primer pair and probe used in the study.

Primers	Sequence	Specificity
dnaJ f420	GTGAAGGGACGGGTGCTAAG	*Vibrio aestuarianus*
dnaJ r456	CCATGACAAGTGCCACAAGTCT	
dnaJ p441 (probe)	FAM-AGGGCACGTCGGC-MGB	

Direct Sanger sequencing was carried out on representative PCR products amplified from cockle samples, seabird faecal and sediment samples, using generic *Vibrio* primers to identify the species present in the samples. Genomic DNA from 10 selected cockle individuals and 11 faecal and sediment samples 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 (Source Bioscience). Each sequence was matched against the National Center for Biotechnology Information (NCBI) nucleotide database with BLASTn (Basic Local Alignment Search Tool), which finds regions of local similarity between sequences to identify and confirm the DNA being detected in the PCRs.

5.2.5. Statistical analysis

Statistical analysis was performed in R version 3.2.3. statistical software. *Vibrio* occurrence was modelled as pathogen presence/absence in each cockle, bird faecal sample and sediment sample. Pearson's Chi-squared tests were used to determine whether the presence of *Vibrio* spp. was significantly different ($p(Chisq) < 0.05$) among the sample sites and throughout the year for each of the sample types (cockles, bird faecal samples and sediment). Fisher's exact test was conducted when the frequencies of the pathogen occurrence in a site or season were < 4, to gain accuracy.

Collinearity between the different compartments - sediment, cockles, and shorebirds - was examined by pairs using non-parametric Kendall's Rank Correlation Tau (τ) with the *Vibrio* occurrence data in each compartment ($p(\tau) < 0.05$). The correlation ($p(\tau) < 0.05$) between the presence of the *C. edule* DNA and *Vibrio* DNA was also examined within two of the compartments, shorebirds and sediment.

5.3. Results

5.3.1. Bird species and observational studies

A detailed list of the bird species identified as well as the behaviour observed in the recording is provided in Supplementary Table S1, likewise, their diet and status are described in Supplementary Table S2.

Overall, the bird presence on the sampled cockle beds, in the intertidal and subtidal areas, was constant at the sample sites and throughout the year (Supplementary Table S1). The only exception was in spring 2019 in Annagassan, where there was a clear disturbance in the shorebird community due to a flying drone in the area. A variety of waders (Supplementary Table S1) - oystercatchers *Haematopus ostralegus*, curlews *Numenius arquata*, bar-tailed godwits *Limosa lapponica*, black-tailed godwits *Limosa limosa*, common redshanks *Tringa totanus*, red knot *Calidris canutus* and dunlins *Calidris alpina* - that feed on bivalves, including cockles, were identified in the recordings along with several species of seagulls - *Larus canus, Chroicocephalus ridibundus, Larus marinus, Larus melanocephalus* - and hooded crows (*Corvus cornix*), both groups with a generalist diet but that can feed on bivalves in coastal areas (Supplementary Table S2). Most waders observed were actively foraging and feeding (Table 4; Supplementary Table S1). Some recording was done of birds, especially oystercatchers, digging out shellfish from the sediment and opening them with their beak. Gulls, in turn, were usually loafing, preening, and passively foraging and feeding (Table 4; Supplementary Table S1).

The sample sites with lower bird occurrence were Ringaskiddy and Youghal, where a few scattered bird individuals were recorded (mainly *H. ostralegus*, *N. arquata* and *L. canus*), except for a flock of *C. canutus* ($10 \leq 20$ individuals) in summer 2018 at Youghal (Table 4; Supplementary Table S1). Bird flocks (>10 individuals) were common in Cuskinny, Dungarvan, and Dundalk (Annagassan and Cooley) (Table 4; Supplementary Table S1). Large gull flocks (>20 individuals) were recorded at Cooley and, especially, at Cuskinny and Dungarvan (Table 4; Supplementary Table S1). In turn, wader flocks ($10 \leq 30$ individuals), including *H. ostralegus, L. limosa, T. totanus* and *C. alpina*, were more frequently recorded in Dundalk sites (Annagassan and Cooley) (Table 4; Supplementary Table S1). Gulls were recorded over the year, except for the winter season in Annagassan and Cooley,

while waders seemed to be more frequently observed during autumn and winter (Supplementary Table S1), which would correlate with their status as winter visitors to Ireland (Supplementary Table S2).

Table 4: The most abundant shorebird species, number, location and behaviour observed at each sample site by season, compiling the main flocks observed in the field.

Sites	Sampling dates	Tidal zonation	Bird species	Number of individuals	Behaviour *
Ringaskiddy	**Spring 2018**	Intertidal	Common gulls (*Larus canus*)	5	O
	Autumn 2018	Intertidal	Oystercatchers (*Haematopus ostralegus*)	3	F
	Winter 2018/19	Intertidal	Oystercatchers (*Haematopus ostralegus*)	5	F
	Spring 2019	Intertidal	Common gulls (*Larus canus*)	2	O
			Great black-backed gulls (*Larus marinus*)	2	
Cuskinny	**Spring 2018**	Intertidal	Hooded crows (*Corvus cornix*)	Flock (~10)	F/O
	Autumn 2018	Intertidal	Black-headed gull (*Chroicocephalus ridibundus*)	Flock (40-50)	F/O
	Winter 2018/19	Intertidal	Common gulls (*Larus canus*)	Flock (20-30)	F/O
			Hooded crows (*Corvus cornix*)	Flock (10-20)	
	Spring 2019	Intertidal	Black-headed gull (*Chroicocephalus ridibundus*)	Flock (30-50)	F/O
Youghal	**Summer 2018**	Intertidal	Red knot (*Calidris canutus*)	Flocks (10-20)	F
	Autumn 2018	Intertidal / subtidal	Common gulls (*Larus canus*)	4	O
	Winter 2018/19	Intertidal / subtidal	Oystercatchers (*Haematopus ostralegus*)	4	F
	Spring 2019	Intertidal	Oystercatchers (*Haematopus ostralegus*)	3	F
Dungarvan	**Summer 2018**	Intertidal	Oystercatchers (*Haematopus ostralegus*)	4	F
	Autumn 2018	Intertidal	Black-headed gull (*Chroicocephalus ridibundus*)	Flock (20-30)	F/O
	Winter 2018/19	Intertidal	Common gulls (*Larus canus*)	Flock (20-30)	O
			Great black-backed gulls (*Larus marinus*)	Flock (20-30)	
	Spring 2019	Intertidal	Oystercatchers (*Haematopus ostralegus*)	Flocks (~10)	F
Annagassan	**Summer 2018**	Intertidal	Dunlins (*Calidris alpina*)	Flock (10-20)	F
	Autumn 2018	Intertidal	Oystercatchers (*Haematopus ostralegus*)	Flocks (~10)	F
			Common redshank (*Tringa totanus*)	Flocks (~10)	
	Winter 2018/19	Intertidal	Oystercatchers (*Haematopus ostralegus*)	Flocks (10-20)	F
			Black-tailed godwits (*Limosa limosa*)	Flocks (10-20)	
	Spring 2019	Supratidal	Raven (*Corvus corax*)	1	F
Cooley	**Summer 2018**	Intertidal	Oystercatchers (*Haematopus ostralegus*)	Flocks (~10)	F
	Autumn 2018	Intertidal	Black-tailed godwits (*Limosa limosa*)	Flock (20-30)	F
	Winter 2018/19	Intertidal	Oystercatchers (*Haematopus ostralegus*)	Flocks (~10)	F
	Spring 2019	Intertidal / subtidal	Black-tailed godwits (*Limosa limosa*)	Flock (20-30)	F
			Common gulls (*Larus canus*)	Mixed flock (>50)	F/O
			Mediterranean gulls (*Larus melanocephalus*)		
			Sandwich terns (*Sterna sandvicensis*)		

(*) Following the descriptions in Lewis and Tierney (2014), shorebird behaviour was classified into two categories: (F) foraging/feeding: the active or passive search of food and feeding; and (O) other: all other behaviours that do not assign to foraging, including roosting, standing, preening, loafing, swimming and others.

5.3.2. Molecular cockle species identification

The examination of the quantity and quality of the extracted DNA from a subsample (n=120) of the collected cockles revealed that all tested samples showed a good DNA quantity, with a mean quantity of 304.13 ng/µl (range between 76.35 ng/µl and 1305.47 ng/µl). Among the subsample, 90% (n=108/120) of the samples had a DNA quantity higher than 100 ng/µl. Likewise, all the tested samples showed high-quality DNA, with a mean of A260/A280 = 1.77 and a range between 1.6 and 2.02. All the screened cockles were identified as *Cerastoderma edule*, with no presence of *Cerastoderma glaucum* or hybrids.

Among the faecal and sediment samples taken, 61.8% (n=126/204) of the faecal samples and 63.2% (n=129/204) of the sediment samples showed good DNA quantity (≥1 ng/µl) and high-quality DNA (A260/A280 = 1 ≤ 1.99), while 17.6% (n=36/204) of the faecal samples and 18.1% (n=37/204) of the sediment samples showed good DNA quantity (≥1 ng/µl) and presence of RNA along with DNA (A260/A280 = 2 ≤ 4).

In the seabird faecal samples, 22.5% (n=46/204) contained *C. edule* DNA (Figure 2), with 63.0% (n=29/46) of those samples displaying high quantities of DNA (≥1 ng/µl) and high-quality DNA (A260/A280 = 1 ≤ 1.99). In the sediment samples, 33.8% (n=69/204) contained *C. edule* DNA (Figure 2), with 56.5% (n=39/69) of those samples displaying good quantities of DNA (≥1 ng/µl) of high-quality (A260/A280 = 1 ≤ 1.99). There was no presence of *C. glaucum* DNA or hybrids in any seabird faecal or sediment samples.

The highest percentage of *C. edule* DNA detected in faecal samples occurred at Dundalk Bay – Annagassan (30.6%; n=11/36) and Cooley (30.0%; n=12/40) –, where the cockle abundance found was high compared to the other sites and where oystercatchers *H. ostralegus*, curlews *N. arquata*, black-tailed godwits *L. limosa*, common redshanks *T. totanus* and dunlins *C. alpina* were observed foraging (Figure 3). Cuskinny, where gulls were the dominant species recorded and the cockle abundance observed in that area was lower than other sites, was the site with the lowest level of *C. edule* DNA found in faeces (12.5%; n=4/32) (Figure 3). Youghal, with a low cockle and bird abundances observed, also showed a low detection of *C. edule* DNA in faeces (14.7%; n=5/34) (Figure 3). The lowest *C. edule* DNA detection in the

sediment samples was in Ringaskiddy (20.6%; n=7/34), where a low abundance of cockles and empty cockle shells were found at every sampling, while the highest detection was in the commercial fishery of Dundalk - Annagassan (36.1%; n=13/36) and Cooley (60.0%; n=24/40) -, with higher cockle abundances observed (Figure 3).

Seasonal variability in the detection of *C edule* DNA in faecal and sediment samples was observed, with the highest detection of *C edule* DNA during spring 2018 (faeces: 100%, n=6/6; sediment: 83.3%, n=5/6) and summer 2018 (faeces: 73.9%, n=17/23; sediment: 78.3%, n=18/23), when more screened cockles were found dead or in poor condition (full of sand and bad smell); while the lowest detection was in winter 2018/19 (faeces: 6.7%, n= 4/60; sediment: 20.0%; n=12/60) (Figure 4). Flocks of *H. ostralegus, C. alpina* and *C. canutus* ($10 \leq 20$ individuals) were observed during summer, while *H. ostralegus*, *T. tetanus*, and *L. limosa* flocks ($10 \leq 30$ individuals) along with large flocks of gulls ($20 \leq 50$ individuals) and hooded crows ($10 \leq 20$ individuals) were observed in autumn and winter (Figure 4).

5.3.3. Pathogen screening

During the study two pathogen groups were detected in *C. edule* samples: 37.7% (n=277/735) tested positive for Haplosporidia, while 25.3% (n=186/735) tested positive for *Vibrio* spp. (Figure 2; Table 5). Of the 277 cockles positive for Haplosporidia, 29.6% (n=82/277) could be distinguished as being infected with *Minchinia tapetis* and 14.8% (n=41/277) were infected with *Minchinia mercenariae*-like (Table 5). However, other haplosporidian species not targeted by this study could also be present in the *C. edule* samples that were positive in the generic haplosporidian PCR. Of the 186 cockles positive for *Vibrio*, 19.9% (n=37/186) were determined to be infected with *Vibrio aestuarianus* (Table 5), with highly infected individuals defined as those individuals that showed a $C_t < 30$, since the *V. aestuarianus* positive controls (diluted purified DNA) had a C_t=30 (60 copies of *V. aestuarianus* DNA/µL). Based on that, 13.5% (n=5/37) of the individuals determined to be positive for *V. aestuarianus* were classified as heavily infected individuals, while 86.5% (n=32/37) were classified as having light/low infections. The mean PCR efficiency value obtained were 100,5% ± 2,72%, and -3,31 ± 0,07% for slope value. Dungarvan Harbour was the site with the highest presence of *V. aestuarianus* and three out of the five heavily infected individuals were found at that site during summer 2018.

Sequencing was conducted with a subsample of the other 80.1% (n=149/186) of samples that were not positive for *V. aestuarianus*, with results listed below (Table 6). No ostreid herpesvirus-1 microVar nor Microsporidia spp. were detected in any of the *C. edule* screened during the study (Table 5).

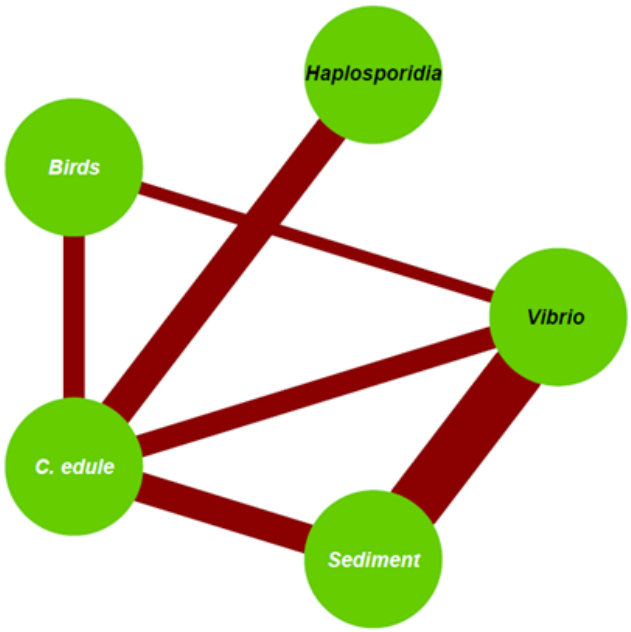


Figure 2: Network association plot displaying the links between the different compartments studied. The black text refers to pathogens and the white text relates to hosts/reservoirs. A solid line represents target DNA presence and line thickness indicates the prevalence (%) of infected *C. edule* and the percentage of positive faecal and sediment samples.

Table 5: Total numbers of positive cases (%, positive individuals/screened individuals) for the screening done for the target pathogen species in cockles, seabird faecal samples and sediment samples.

Pathogen species	Cockle samples	Seabird faecal samples	Sediment samples
Haplosporidia	**37.7% (277/735)**	0% (0/84)	0% (0/84)
Minchinia tapetis	**29.6% (82/277)**	0% (0/84)	0% (0/84)
***Minchinia mercenariae*-like**	**14.8% (41/277)**	0% (0/84)	0% (0/84)
Ostreid herpesvirus type-1 and variants	0% (0/735)	0% (0/113)	0% (0/113)
Microsporidia sp.1	0% (0/735)	0% (0/58)	0% (0/58)
Microsporidia sp.2	0% (0/735)	0% (0/58)	0% (0/58)
***Vibrio* spp.**	**25.3% (186/735)**	**13.7% (28/204)**	**62.7% (128/204)**
Vibrio aestuarianus	**19.9% (37/186)**	0% (0/204)	**1.6% (2/204)**

Of the various pathogen groups screened for, only vibrios were found in both bird faecal samples and sediment, apart from in *C. edule* samples (Figure 2). *Vibrio* spp. DNA was detected in 13.7% of the seabird faeces (n=28/204) and 62.7% (n=128/204) of the sediment samples (Table 5). 64.3% (n=18/28) of those faecal samples and 66.4% (n=85/128) of those sediment samples displayed good quantities of DNA (≥1 ng/µl) and high-quality DNA (A260/A280 = 1 ≤ 1.99). The PCR-positive samples for generic *Vibrio* in the three screened sample types were defined as *Vibrio* spp. for analysis purposes. However, specific screening of *V. aestuarianus* was conducted by qPCR and sequencing done with subsamples of the *C. edule*, faecal and sediment samples revealed other *Vibrio* species present during the study but not quantified.

V. aestuarianus was absent in the faecal samples, and it was only detected in two of the sediment samples (1.6%, n=2/204), in spring 2018 at Cuskinny and in summer 2018 at Dungarvan (Table 5), and both were classified as having low bacteria loading. PCR efficiency ranged between 97% and 99%, with corresponding slope values between -3.2 and -3.3. The strain of *V. aestuarianus* detected in cockles and sediment was confirmed to be the same (MK307696.1, strain 15_075_1T2), which has been recently confirmed to be pathogenic to the common cockles *C. edule* (Garcia et al., 2021). Other species of *Vibrio*, known as important aetiological agents of diseases affecting all life stages of shellfish (Gay et al., 2004; Romalde et al., 2014), were also identified: *Vibrio splendidus* and *Vibrio kanaloae*. It was confirmed that three strains of *V. splendidus* identified by direct Sanger sequencing were the same in the three sample types (Table 6). The sequences obtained from the three samples types, with a query length between 87-155bp, showed 60-91% query coverage and 92.6-98.7% percent identity to *V. splendidus* strains deposited by Gao (2020) (MT445179.1, strain FS1; MT445177.1, strain CT1) and by Landreau et al. (2020) (MT345091.1, strain D0-B01). Some of these sequences obtained from *C. edule* and bird faecal samples came from the same sampling site, Dungarvan (Table 6). However, the identified sequences from the sediment are from Ringaskiddy. Likewise, the *V. kanaloae* strain CAIM 485 was found by sequencing in both cockle and sediment samples (Table 6). The cockle samples, with a query length between 82-179bp, showed 78-83% query coverage and 92.6-98.6% percent identity to *V. kanaloae* deposited by Sun (2020) (MT759943.1); while the sequence from the

sediment sample, with a query length of 222bp, showed 30% query coverage and 94.4% percent identity to *V. kanaloae* deposited by Sun (2020) (MT759943.1). Nevertheless, it was not considered a robust outcome due to the low query cover (30%) in the sequenced sediment sample. For two of the sequenced cockle samples, it was not possible to generate sequences that were long enough to identify species.

Table 6: Description of the Blast results obtained from the sequenced DNA of cockles, seabird faeces and sediment samples using generic *Vibrio* primers.

Sample type	Sample site	Season	Species identification	Percent Identity	Query Cover	Query Length (bp)
Cockles	Youghal Dungarvan Cooley	Summer 2018	*Vibrio spp.* among them: *Vibrio splendidus** and *V. kanaloae**	92.59-98.58%	78-83%	82-179
Cockles	Annagassan Annagassan	Summer 2018	*Vibrio spp*. most likely *V. splendidus**	97.84-97.92%	88-91%	154-155
Cockles	Annagassan	Summer 2018	*Vibrio spp.* most likely *V. aestuarianus* or *V. mediterranei*	98.01%	94%	158
Cockles	Cuskinny Dungarvan	Autumn 2018	*Vibrio spp.*	94.23-94.29%	67-73%	52-69
Sediment	Cooley Dungarvan	Summer 2018	*Vibrio spp.*	95.31-95.83%	85-93%	74-75
Sediment	Ringaskiddy	Autumn 2018	*Vibrio splendidus*	92.73%	85%	62
Sediment	Ringaskiddy Ringaskiddy	Autumn 2018 Winter 2018	*Vibrio sp*. most likely *V. splendidus**	95.65-98.51%	60%	110-114
Sediment	Youghal	Summer 2018	*Vibrio sp.* most likely *V. kanaloae**	94.37%	30%	222
Shorebird faeces	Dungarvan	Spring 2019	*Vibrio sp*. most likely *V. splendidus**	98.67%	86%	87
Shorebird faeces	Cooley	Summer 2018	*Grimontia hollisae (previously classified as Vibrio hollisae)*	97.14%	50%	138
Shorebird faeces	Youghal	Spring 2019	*Vibrionaceae bacterium*	100%	80%	57
Shorebird faeces	Annagassan	Autumn 2018	Enterobacter/Kiebsiella spp.	96.43%	73%	75
Shorebird faeces	Cuskinny	Autumn 2018	*Enterobacterales*	98.48%	44%	146

(*) Same strains of Vibrio spp. were identified in cockle samples and sediment/faecal samples.

The prevalence of *Vibrio* spp. displayed significant spatial variability ($p(Chisq)<0.05$) in both *C. edule* and bird faecal samples (Figure 3), although, no significant correlation ($p(\tau) > 0.05$) in the prevalence of *Vibrio* spp. was established between cockles and shorebird compartments. Ringaskiddy displayed the lowest prevalence of *Vibrio* spp. (2.9%; n=1/34) in the bird faecal samples, but also showed

a low prevalence of *Vibrio* spp. in *C. edule* samples (13.7%; n=16/117) (Figure 3). In turn, Youghal, Annagassan and Cooley showed intermediate values in the prevalence of *Vibrio* spp. detected in *C. edule* samples and bird faecal samples (Figure 3). Cuskinny was the site showing the lowest prevalence of *Vibrio* spp. in cockle samples (10.7%; n=15/140), while Dungarvan presented the highest prevalence (44.4%; n=75/169) (Figure 3). In contrast, the highest prevalence of *Vibrio* spp. detected in faecal samples was in Cuskinny (31.3%; n=10/32), with the lowest level of *C. edule* DNA found in the faeces of that area (12.5%; n=4/32) (Figure 3); while Dungarvan was the second site with the lowest prevalence of *Vibrio* spp. in faecal samples (7.1%; n=2/28) and a low level of *C. edule* DNA found in the faeces (17.9%; n=5/28) (Figure 3). No significant correlation ($p(\tau) > 0.05$) was established between the presence of *C. edule* DNA and *Vibrio* DNA detected within the shorebird compartment.

The presence of *Vibrio* spp. in sediment samples overpassed the prevalence of *Vibrio* spp. in bird faecal samples and to a lesser extent in *C. edule* samples (Figure 3). The examination of collinearity in the prevalence of *Vibrio* spp. between the different compartments - sediment, cockles, and shorebirds – revealed a correlation ($\tau > 0.3$) between sediment and cockles.

In sediments samples, no significant differences in *Vibrio* presence between sample sites were displayed ($p(Chisq) > 0.05$). The occurrence of *Vibrio* spp. remained fairly high through the sites, regardless of the different levels of *C. edule* DNA found in the sites - no significant correlation ($p(\tau) > 0.05$) was established between the presence of *C. edule* DNA and *Vibrio* DNA detected within the sediment compartment - (Figure 3).

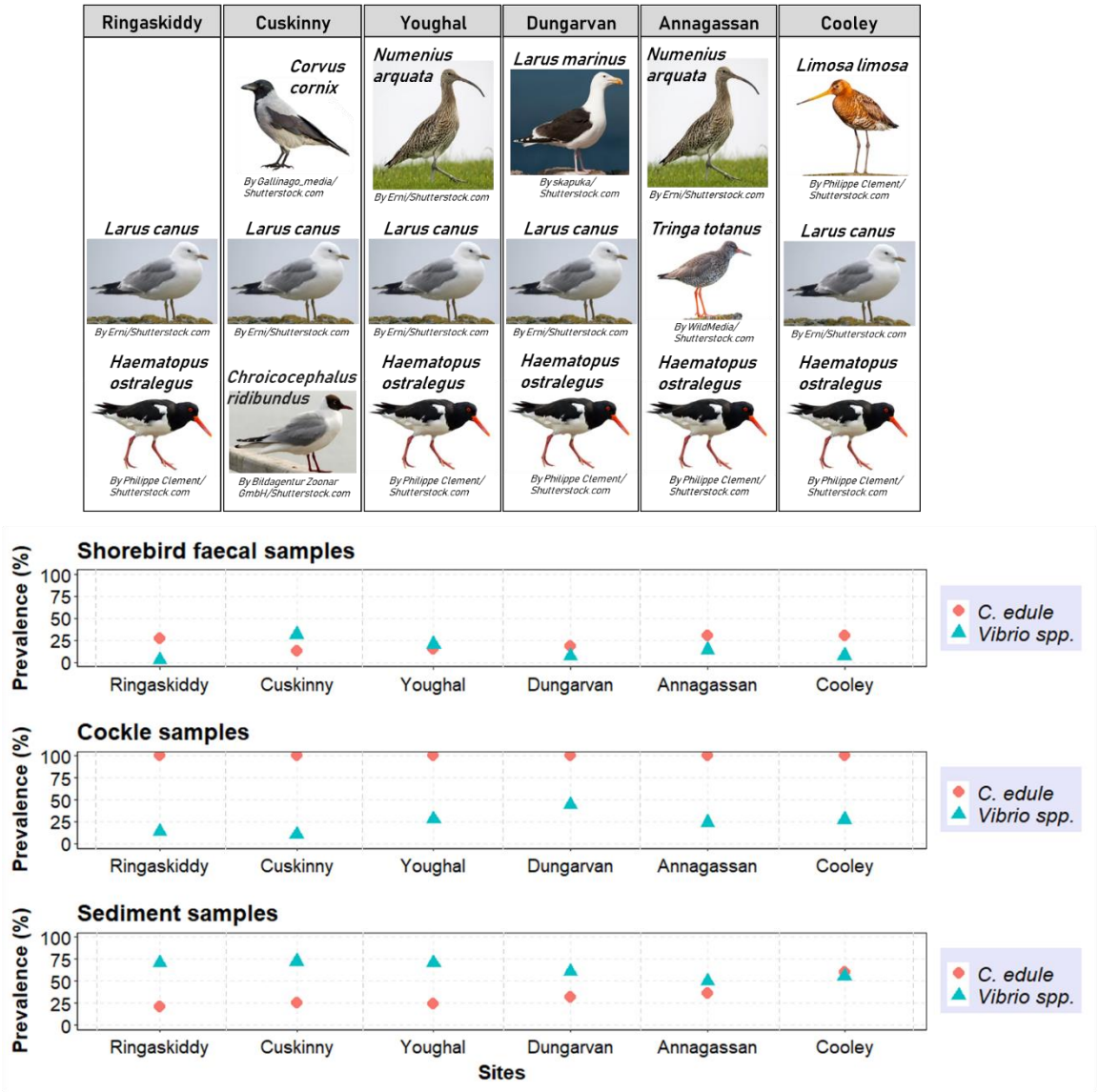


Figure 3: Distribution of *C. edule* DNA compared to the presence of *Vibrio* spp. in shorebird faecal samples, cockles, and sediment samples at each sample site. The most frequently observed shorebird species throughout the seasons at each sample site are represented on the top of the figure (Images under license from Shutterstock.com).

Significant seasonal variability ($p(Chisq) < 0.01$) in the occurrence of *Vibrio* spp. was exhibited in the three studied compartments – sediment, cockles, and shorebirds-. The highest presence of *Vibrio* spp. in *C. edule*, bird faecal samples and sediment samples, was during the warmer months and declined during the winter (Figure 4). The presence of *Vibrio* spp., therefore, followed the same seasonal trend as the occurrence of *C. edule* DNA in both faeces and sediment samples (Figure 4), although the correlation between *C. edule* DNA and *Vibrio* DNA detected in those compartments was not statistically significant ($p(\tau) > 0.05$). 28.6% (n=8/28) of the

faecal samples that were positive for *Vibrio* spp. also contained *C. edule* DNA and all of them were from spring and summer. It was also noticeable that in spring 2019 the prevalence of infection was higher in the three compartments compared to spring 2018 (Figure 4) when the only samples available were from Ringaskiddy and Cuskinny.

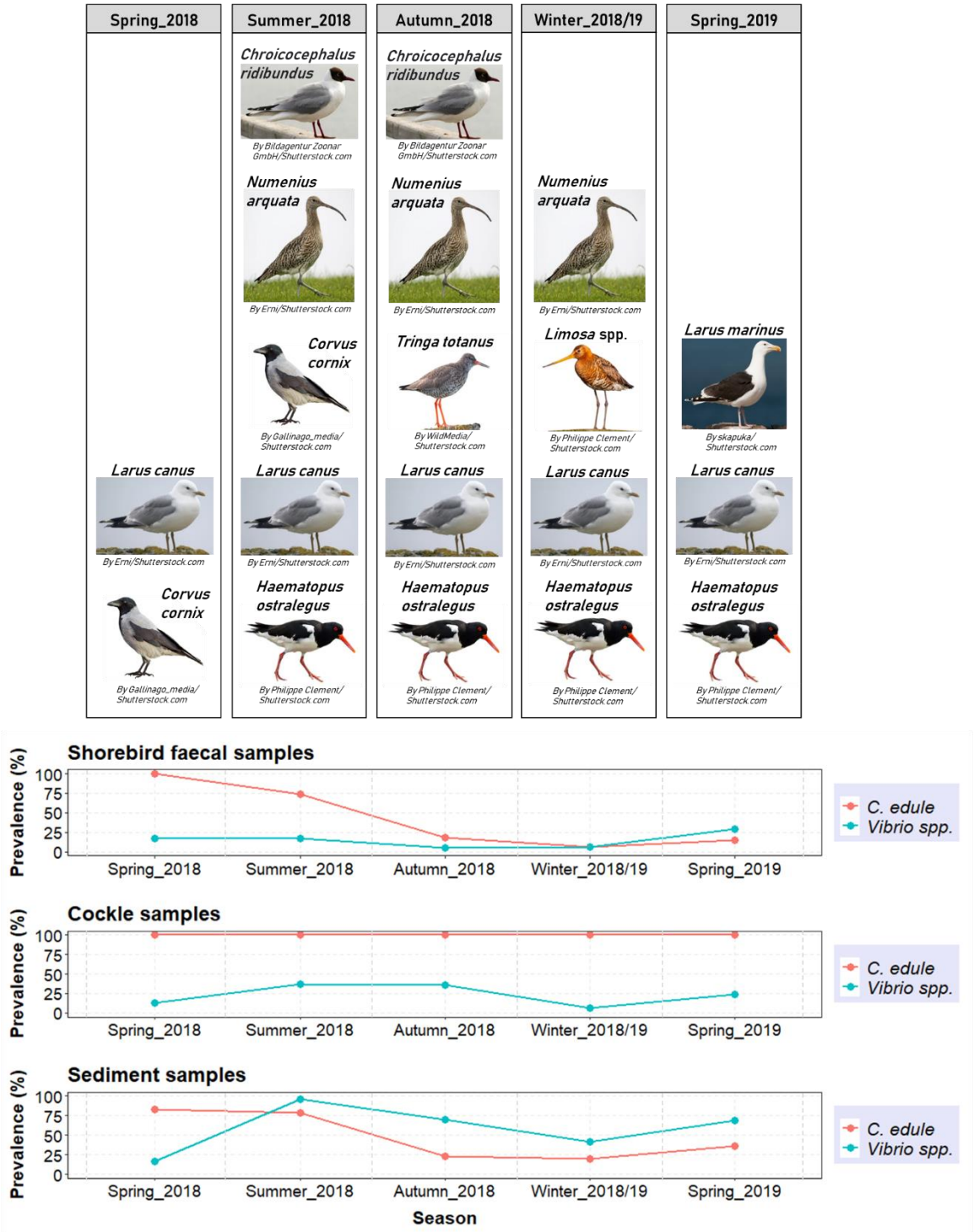


Figure 4: Distribution of *C. edule* DNA compared to the presence of *Vibrio* spp. in shorebird faecal samples, cockles and sediment samples throughout the seasons. The most frequently observed shorebird species through the sites by each season are represented on the top of the figure (Images under license from Shutterstock.com).

5.4. Discussion

This study demonstrates connectivity between the presence of vibrios in the marine environment and the different compartments that they can be detected in, i.e., sediment, invertebrates, and vertebrates. The presence of *Vibrio* spp. was confirmed in sediment samples, that may act as a reservoir or sink for such bacteria; in sediment-dwellers *C. edule*, where some may be pathogenic; and in shorebirds, infected via the consumption of cockles, resulting in transmission of these bacteria via faeces and the further repopulating of the sediment by these faecal bacteria. Unlike *Vibrio* spp., haplosporidians, including *Minchinia* spp., were found in *C. edule* samples but no transmission to shorebirds was detected. Haplosporidia DNA was also absent in the sediment. The lack of detection of haplosporidians supports the effectiveness of the *Vibrio* pathogen group to be transferred through the bird digestive system and persist in the environment. This finding may provide new insight into the life cycle of haplosporidians, which is still not fully known, and its capacity of persistence outside the host. However. further research into the infective stages of these pathogens is needed.

Two *Vibrio* species were identified that were of interest due to their pathogenicity and their major role in bivalve disease outbreaks (Lacoste et al., 2001; Le Roux et al., 2002; Waechter et al., 2002; Garnier et al., 2007; Garcia et al., 2021); *Vibrio splendidus,* known as an important aetiological agent of diseases affecting *Crassostrea gigas* aquaculture (Lacoste et al., 2001; Le Roux et al., 2002), and *Vibrio aestuarianus,* whose harmful effect has been reported on *C. edule* (Garcia et al., 2021) and *C. gigas* (McCleary and Henshilwood, 2015). For the first time, to the best of our knowledge, identical strains of *V. splendidus* have been reported in *C. edule,* faecal samples of wild shorebirds and sediment. *V. aestuarianus* was detected in *C. edule* through the sample sites and in sediment from Cuskinny and Dungarvan, but it was absent in the faecal samples. A potential explanation for this absence is that the bacterial load in cockles was not high enough to be transmitted to the birds during foraging or to be detected due to the low levels present.

V. splendidus detection in bird faeces further supports the carrier role that migratory birds play in the dissemination of *Vibrio* species, such as in *V. cholerae* (Halpern et al., 2008; Rodríguez et al., 2010; Fernández-Delgado et al., 2016; Laviad-

Shitrit et al., 2019), *V. parahaemolyticus, V. vulnificus* and *V. mimicus* (Buck, 1990; Miyasaka et al., 2006; Fu et al., 2019). It is well-known that the dispersal of *V. cholerae* by aquatic birds may be attributable to their direct predation of fish (Senderovich et al., 2010; Laviad-Shitrit et al., 2017; Hossain et al., 2018), chironomids and copepods (Halpern et al., 2008). Recently, Fu et al. (2019) demonstrated that the predation of molluscs and zooplankton by migratory birds played an essential role in the dissemination of *V. parahaemolyticus* and *V. mimicus* in the estuary of the Liaohe River in China. However, the transmission of *Vibrio* species less relevant to humans had been neglected and very little is known about the role that migratory birds play in the spread of these pathogens. Our findings, therefore, provided the first evidence of possible transmission of *V. splendidus* to birds through consumption of infected cockles, as was supported by the presence of *C. edule* DNA and *Vibrio* DNA in the same bird faecal samples. In addition, as further discussed below, the presence of *Vibrio* spp. followed the same seasonal trend in both *C. edule* and faeces samples, likewise, similar outcomes were observed in those two compartments at some of the sites. Moreover, the identified strains of *V. splendidus* in *C. edule* and bird faecal samples came from the same sample site, Dungarvan, with the highest prevalence of *Vibrio* spp. in *C. edule* samples. Those results and the taken measures of sterilisation and correct handling of the stools to minimise the cross-contamination with the sediment provided a solid base for identifying the infected cockles as the source of *V. splendidus* in shorebirds. The examination of collinearity in the prevalence of *Vibrio* spp. between sediment and shorebird faeces, in turn, revealed no correlation between those compartments. The consumption of infected cockles as the source of *V. splendidus* in shorebird faeces was further supported by the observations of waders, mainly oystercatchers *H. ostralegus*, known to feed on bivalves including cockles, at the sample sites. Cockle availability is a key resource supporting many overwintering wader populations such as oystercatchers (Carss et al., 2020). The observational data collected also revealed that the presence of waders was larger at the sample sites with higher cockle abundances observed during the sampling. Previous studies have demonstrated a positive correlation between shorebird abundance and invertebrate prey densities, especially when patterns are examined across large spatial scales (e.g., encompassing entire estuaries) (Bryant, 1979; Hicklin and Smith, 1984; Colwell and Landrum, 1993). In sum, those outcomes

provided robust evidence that the *V. splendidus* was potentially transmitted through the consumption of infected cockles by shorebirds.

The spatial variability in the *Vibrio* infection established in *C. edule* and bird faecal samples added evidence to the influence that cockle consumption may have had on the *Vibrio* prevalence detected in shorebird faecal samples. Wader flocks were common in Dundalk (Annagassan and Cooley) with the highest presence of cockles observed in the field and the highest densities reported in the literature (Shellfish Stocks and Fisheries Review, Marine Institute and BIM, 2017). Both sites in Dundalk showed the highest presence of *C. edule* DNA in the bird faeces, suggesting the waders are feeding on the cockles of those areas. In turn, Annagassan and Cooley showed intermediate values in the prevalence of *Vibrio* spp. detected in both *C. edule* samples and bird faecal samples. Despite the more frequent observation of waders in the sites with easier access to cockles, the correlation in *Vibrio* prevalence between *C. edule* and shorebird compartments was not significant. The strongest discrepancy in the prevalence of *Vibrio* spp. detected between the *C. edule* samples and the faecal samples was found at Cuskinny and Dungarvan. Dungarvan, with the highest prevalence of *Vibrio* spp. and *V. aestuarianus* in *C. edule*, showed a low prevalence of *Vibrio* spp. in bird faecal samples, probably due to the predominant presence of gulls, with a generalist diet, with respect to waders. Cuskinny exhibited the lowest prevalence of *Vibrio* spp. in cockle samples and the highest prevalence of *Vibrio* spp. in faecal samples, being also the gulls the dominant birds present. Therefore, it may reflect a lower consumption of cockles by gulls. This is consistent with the lower presence of *C. edule* DNA found in bird faecal samples at both sites. In the case of Cuskinny, the high prevalence of *Vibrio* spp. in faecal samples may be due to another source of *Vibrio* rather than the consumption of cockles.

Seasonal variability in the *Vibrio* infection was confirmed in *C. edule* and bird faecal samples and may also be related to the cockle consumption by shorebirds. Prevalence of *Vibrio* spp. increased in *C. edule* and faecal samples during the warmer months, probably due to the higher temperature. Temperature is likely the most important driver of the overall change in *Vibrio* abundance in temperate coastal waters (Vezzulli et al., 2015). Temperature promotes the proliferation of bacteria such as *Vibrio*, which grow preferentially in warm waters (Vezzulli et al., 2013; Vezzulli et al., 2015). The high seawater temperature may also have an important impact on the

physiological state of bivalve hosts, which makes them more susceptible to infection during the warm period of the year (Ben-Horin et al., 2015). Consequently, infected *C. edule* may have been more accessible for shorebirds at that time of the year, when more screened cockles were found in poor condition. This higher susceptibility of cockles may have also promoted its opportunistic consumption by generalist feeders such as gulls and hooded crows, as suggested by the higher occurrence of *C. edule* DNA detected during the warmer months in the bird faecal samples. Although no significant correlation was established between the presence of *C. edule* DNA and *Vibrio* DNA in faecal samples, *Vibrio* prevalence followed the same seasonal trend as the occurrence of *C. edule* DNA in bird faeces. It was also noticeable that in spring 2019 the prevalence of infection was higher in *C. edule* and faecal samples compared to spring 2018. This might be because the only samples available in spring 2018 were from Ringaskiddy and Cuskinny, where the prevalence of *Vibrio* spp. was low in *C. edule* samples. Besides, in Cuskinny, gulls, which are not exclusively dependent on cockles for their feeding, were most frequently observed.

As expected, due to the infaunal lifestyle, *C. edule* DNA was also detected in sediment samples, with higher frequency than in the faecal samples. The detection of *C. edule* DNA in the sediment samples was higher at the sites with higher cockle abundances observed, i.e. Dundalk (Annagassan and Cooley). The detection of *C. edule* DNA in Ringaskiddy and Youghal was the lowest, as they were the areas where more effort was required to find enough cockles for the study. Empty cockle shells were observed at Ringaskiddy at every sampling, while Youghal is an extremely muddy shore, which makes it less suitable for cockles that prefer a sand-mud mixture best for cockle growth (Carss et al., 2020). A significant correlation between the occurrence of *Vibrio* spp. in cockles and sediment was established, confirming the link between these two compartments. In sediment samples, the total frequency of *Vibrio* spp. detected was higher than in shorebird faeces and *C. edule* samples. Previous studies have shown that attachment to surfaces, such as the exoskeleton of chitin as well as sediment, is an integral part of the aquatic lifestyle of many vibrios, representing a successful survival mechanism (Pruzzo et al., 2008; Vezzulli et al., 2010; Vezzulli et al., 2015; Azandégbé et al., 2010). Vezzulli et al. (2015) observed that both *V. aestuarianus* and *V. splendidus* maintained viability and culturability for longer times in the sediment than in seawater in laboratory experiments, suggesting

that this compartment may represent a suitable niche for their persistence in the environment. Accordingly, Johnson et al. (2010) observed a protective effect in sediment compared with oysters and seawater, for *V. parahaemolyticus* and *V. vulnificus*. Freitas et al. (2020) recently demonstrated that *V. parahaemolyticus* cells attach to underwater surfaces, such as sediment, and/or associate with various species of shellfish and zooplankton as part of their life cycle. In our study, no significant spatial variability was detected in the presence of *Vibrio* spp. in sediment samples. *Vibrio* spp. frequency remained consistently high through the sample sites regardless of the different levels of *C. edule* DNA detected, with no correlation established between the *C. edule* DNA and *Vibrio* DNA found in the sediment. The findings highlight the role of the sediment as a major reservoir for *Vibrio* spp.

Vibrio spp. showed a lower prevalence during winter in both *C. edule* and faecal samples, however, *Vibrio* spp. frequency remained higher in the sediment. Accordingly, Vezzulli et al. (2009) identified the sediment as an environmental reservoir for *Vibrio*, where the bacteria can find a favourable environment for overwintering. This is also probably linked to the fact that sediment provides biotic and abiotic surfaces useful for bacterial biofilm development; moreover, the concentration of organic matter in this compartment is higher than in the overlying water column (10000–100000 fold-higher in natural conditions) (Vezzulli et al., 2009).

The strains of *V. splendidus* found in the sediment were the same as the ones in *C. edule* and bird faecal samples, although the strains of *V. splendidus* in sediment were identified in a different cockle bed, as well as the same strain of *V. aestuarianus* was detected in *C. edule* and sediment samples. In waterborne infections, in fact, the pathogenic agent can be shed by infected migrating birds, resulting in contamination of water with faeces or other corporal discharges (Hubálek, 2004). Subsequently, molluscs inhabiting the area may get infected by filtration of the water column with the pathogenic agent in it (Fu et al., 2019). The presence of pathogens in the sediment may also have an impact on the infaunal community within the sediment, which influence ecosystem services such as bioturbation, nutrient recycling or carbon sequestration (Carss et al., 2020). The presence of the same vibrios in the three analysed compartments exposes, therefore, the intricate links of the marine trophic webs and the influence of the environment on them.

The findings obtained from this research provide new insight into the parasite-inclusive food web studies of mudflat ecosystems, targeting microparasites and pathogens that have received much less attention in this respect. This study highlights that the connectivity existing between different compartments within the food web can be extended to the microparasites and pathogens present. The observed differences in the transmission of the Haplosporidia and the *Vibrio* spp. analysed indicates that not all pathogen groups are transmitted via food webs and that certain pathogens such as bacteria can be ingested and excreted with their DNA integrity intact thus indicating viability. The findings shed light on the potential transmission of *V. splendidus* associated with bivalves to shorebirds, via cockle consumption; as it has been previously reported with oysters (Laviad-Shitrit et al., 2019) and fish (Laviad-Shitrit et al., 2017), whose intake transmits *V. cholerae* to aquatic birds. Our findings add evidence to the significant role migratory birds may have as carriers of infectious agents such as bacteria, enabling their transport and dispersal not only to a short distance, given the efficient digestion and short food retention time in shorebirds, but also to a long distance if the infection in shorebirds occurs (Piersma et al., 1993). Further examination of regurgitated pellets and faecal droppings, as well as the analysis of gut contents of shorebirds, would be necessary to definitively confirm the subsequent trophic transmission and the infection success and impacts this pathogen can have on shorebird populations. Likewise, our findings further support the role of sediment as a *Vibrio* reservoir. The analysis of other compartments in the field, such as seawater, cohabiting bivalves, etc., and the factors affecting the survival of the infectious agents, would be of interest for future research to provide a complete picture of the infection sources and the potential connectivity between them.

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Supplementary Material

Supplementary Table S1: Seabird species, number, location and behaviour observed at each sample site (Ringaskiddy (Ringa.), Cuskinny (Cuski.), Youghal (Youg.), Dungarvan (Dung.), Annagassan (Annag.), and Cooley) by season.

Sites	Sampling dates	Tidal zonation	Bird species	Number of individuals	Behaviour
Ringa.	**Spring 2018**	Intertidal	Common gulls (*Larus canus*)	5	Loafing and swimming.
			Hooded crows (*Corvus cornix*)	1	Passively foraging.
	Autumn 2018	Intertidal	Common gulls (*Larus canus*)	2	Standing and swimming
			Black-headed gull (*Chroicocephalus ridibundus*)	1	
			Oystercatchers (*Haematopus ostralegus*)	3	Actively foraging and feeding
			Common redshank (*Tringa totanus*)	1	
			Curlew (*Numenius arquata*)	1	Flying
	Winter 2018/19	Intertidal	Common gulls (*Larus canus*)	3	Loafing and swimming.
			Oystercatchers (*Haematopus ostralegus*)	5	Actively foraging and feeding
			Curlew (*Numenius arquata*)	1	Passively foraging and preening.
			Brent gooses (*Branta bernicla*)	4	Swimming
	Spring 2019	Intertidal	Common gulls (*Larus canus*)	2	Loafing
			Great black-backed gulls (*Larus marinus*)	2	
Cuski.	**Spring 2018**	Intertidal	Common gulls (*Larus canus*)	4	Loafing
			Hooded crows (*Corvus cornix*)	Flock (~10)	Passively foraging and/or standing.
	Autumn 2018	Intertidal	Black-headed gulls (*Chroicocephalus ridibundus*)	Flock (40-50)	Preening, loafing, and passively foraging and feeding.
			Great black-backed gulls (*Larus marinus*)	2	
			Hooded crows (*Corvus cornix*)	1	Actively foraging and feeding
			Oystercatcher (*Haematopus ostralegus*)	1	
	Winter 2018/19	Intertidal	Common gulls (*Larus canus*)	Flock (20-30)	Passively foraging and/or standing.
			Hooded crows (*Corvus cornix*)	Flock (10-20)	
			Black-headed gulls (*Chroicocephalus ridibundus*)	4	
			Oystercatcher (*Haematopus ostralegus*)	1	Actively foraging and feeding
	Spring 2019	Intertidal	Black-headed gulls (*Chroicocephalus ridibundus*)	Flock (30-50)	Passively foraging and/or standing.
			Common gulls (*Larus canus*)	5	
			Great black-backed gulls (*Larus marinus*)	4	
			Hooded crows (*Corvus cornix*)	8	Actively foraging and feeding.

Youg.	**Summer 2018**	Intertidal	Red knot (*Calidris canutus*)	Flocks (10-20)	Actively foraging and feeding.
			Black-headed gulls (*Chroicocephalus ridibundus*)	2	Loafing
			Oystercatchers (*Haematopus ostralegus*)	2	Actively foraging and feeding.
			Curlews (*Numenius arquata*)	4	
			Grey heron (*Ardea cinerea*)	1	
	Autumn 2018	Intertidal and subtidal	Oystercatchers (*Haematopus ostralegus*)	3	Actively foraging and feeding.
			Common gulls (*Larus canus*)	4	Standing
			Curlew (*Numenius arquata*)	1	Actively foraging and feeding
			Bar-tailed godwits (*Limosa lapponica*)	2	
	Winter 2018/19	Intertidal and subtidal	Curlews (*Numenius arquata*)	2	Actively foraging and feeding.
			Oystercatchers (*Haematopus ostralegus*)	4	
			Bar-tailed godwit (*Limosa lapponica*)	1	
			Common gulls (*Larus canus*)	2	Loafing.
	Spring 2019	Intertidal	Oystercatchers (*Haematopus ostralegus*)	3	Actively foraging and feeding.
			Common gulls (*Larus canus*)	2	Standing.
			Grey heron (*Ardea cinerea*)	1	Actively foraging.
Dung.	**Summer 2018**	Intertidal	Common gull (*Larus canus*)	1	Passively foraging and feeding.
			Oystercatchers (*Haematopus ostralegus*)	4	
			Hooded crow (*Corvus cornix*)	1	Standing
	Autumn 2018	Intertidal	Common gulls (*Larus canus*)	6	Passively foraging and/or standing.
			Great black-backed gulls (*Larus marinus*)	6	
			Black-headed gulls (*Chroicocephalus ridibundus*)	Sparse individuals (8) A flock (20-30)	
			Hooded crow (*Corvus cornix*)	1	
			Oystercatchers (*Haematopus ostralegus*)	4	Actively foraging and feeding.
			Common redshanks (*Tringa totanus*)	9	
	Winter 2018/19	Intertidal	Common gulls (*Larus canus*)	Flock (20-30)	Loafing
			Great black-backed gulls (*Larus marinus*)	Flock (20-30)	
	Spring 2019	Intertidal	Common gulls (*Larus canus*)	7	Loafing and preening.
			Great black-backed gulls (*Larus marinus*)	1	
			Oystercatchers (*Haematopus ostralegus*)	Flocks (~10)	Actively foraging and feeding.
			Brent gooses (*Branta bernicla*)	10	Swimming, loafing and preening.

Annag.	**Summer 2018**	Intertidal	Oystercatchers (*Haematopus ostralegus*)	9	Actively foraging and feeding.
			Curlews (*Numenius arquata*)	3	
			Black-headed gulls (*Chroicocephalus ridibundus*)	7	
			Dunlins (*Calidris alpina*)	Flock (10-20)	
			Common gulls (*Larus canus*)	3	Passively foraging and/or standing.
			Hooded crows (*Corvus cornix*)	1	
	Autumn 2018	Intertidal	Oystercatchers (*Haematopus ostralegus*)	Flocks (~10)	Actively foraging and feeding.
			Common redshank (*Tringa totanus*)	Flocks (~10)	
			Curlew (*Numenius arquata*)	1	
			Little egret (*Egretta garzetta*)	1	
			Common gulls (*Larus canus*)	1	Loafing.
	Winter 2018/19	Intertidal	Oystercatchers (*Haematopus ostralegus*)	Flocks (10-20)	Actively foraging and feeding.
			Black-tailed godwits (*Limosa limosa*)	Flocks (10-20)	
			Common redshank (*Tringa totanus*)	1	
			Curlew (*Numenius arquata*)	1	
	Spring 2019	Supratidal	Raven (*Corvus corax*)	1	Actively foraging
Cooley	**Summer 2018**	Intertidal	Common gulls (*Larus canus*)	4	Standing and loafing
			Black-headed gulls (*Chroicocephalus ridibundus*)	2	
			Great black-backed gull (*Larus marinus*)	1	
			Hooded crows (*Corvus cornix*)	2	
			Black-tailed godwits (*Limosa limosa*)	3	Actively foraging and feeding.
			Oystercatchers (*Haematopus ostralegus*)	Flocks (~10)	
	Autumn 2018	Intertidal	Common gulls (*Larus canus*)	3	Loafing and standing.
			Black-headed gulls (*Chroicocephalus ridibundus*)	2	
			Black-tailed godwits (*Limosa limosa*)	Flock (20-30)	Actively foraging and feeding.
			Grey heron (*Ardea cinerea*)	1	Standing
			Little egret (*Egretta garzetta*)	1	Passively foraging.
	Winter 2018/19	Intertidal	Oystercatchers (*Haematopus ostralegus*)	Flocks (~10)	Actively foraging and feeding.
			Black-tailed godwits (*Limosa limosa*)	5	
	Spring 2019	Intertidal and subtidal	Black-tailed godwits (*Limosa limosa*)	Flock (20-30)	Actively foraging and feeding.
			Common gulls (*Larus canus*)	Mixed flock (>50)	Actively foraging and feeding. Some loafing and preening.
			Mediterranean gulls (*Larus melanocephalus*)		
			Sandwich terns (*Sterna sandvicensis*)		

Supplementary Table S2: List of bird species observed in the study, their diet and status (based on www.birdwatchireland.ie).

Bird species	Diet	Status
Oystercatcher (*Haematopus ostralegus*)	The main food resource includes the larger invertebrates, particularly mussels and **cockles**.	Resident and winter visitor from Iceland and the Faeroes.
Curlew (*Numenius arquata*)	Feed mostly on invertebrates, particularly ragworms, crabs and molluscs including **cockles** (Goss-Custard and Jones, 1976).	Winter visitor to wetlands, as well as breeding in small numbers in floodplains and boglands.
Bar-tailed godwit (*Limosa lapponica*)	Polychaete worms, particularly lugworms, form a large proportion of their diet. On the muddier estuaries, where lugworms may be absent, they take ragworms and **bivalves**.	Winter visitor to coastal estuaries from Russia and Scandinavia
Black-tailed godwit (*Limosa limosa*)	Feed on a range of invertebrates, including **bivalves**, polychaete worms and shore crabs.	Winter visitor from Iceland.
Red knot (*Calidris canutus*)	Feed predominantly on small bivalves, especially mussels, clams and **cockles**, and also on crustaceans.	Winter visitor from northern Greenland and the Queen Elizabeth Islands of high Artic Canada west to Prince Patrick Island. In spring and light summer some breeding plumage birds can be spotted.
Dunlin (*Calidris alpina*)	Feed predominantly on small invertebrates of estuarine mudflats, particularly polychaete worms and small molluscs, including **cockles** (Dit Durell and Kelly, 1990).	Summer visitor from NW Africa/SW Europe, winter visitor from Scandinavia to Siberia, passage migrant from Greenland (heading south to winter in Africa).
Common redshank (*Tringa totanus*)	Feed mainly on insects, earthworms, crustaceans and molluscs, including **cockles** (Goss-Custard and Jones, 1976).	Resident, winter visitor from Iceland and passage migrant (from Scandinavia/the Baltic breeding areas to African wintering areas).
Common gull (*Larus canus*)	Feed on a wide range of terrestrial and aquatic insects and invertebrates, also fish, carrion and rubbish. On sandy and muddy shores molluscs, including **cockles**, can serve as food (Vernon, 1972).	Resident and winter visitor from Europe.
Black-headed gull (*Chroicocephalus ridibundus*)	Feed on mostly animal material, including a wide variety of insects, also earthworms, marine worms, molluscs, crustaceans, small fish and carrion. On sandy and muddy shores molluscs, including **cockles**, can serve as food (Vernon, 1972).	Resident along all Irish coasts, with significant numbers arriving from the Continent in winter.
Great black-backed gull (*Larus marinus*)	Diet includes carrion, fish, molluscs, crustaceans, marine worms, insects, rodents, berries, and the adults, young, and eggs of other birds. On mudflats, they follow the retreating tide to capture worms and small **bivalves**.	Resident along all Irish coasts.
Mediterranean gull (*Larus melanocephalus*)	Feed on terrestrial and aquatic insects, marine molluscs, including **bivalves,** and fish, also offal and carrion.	Breeds in small numbers in the southeast. Winter visitor from northwest France, Belgium and the Netherlands.

Sandwich tern (*Sterna sandvicensis*)	Feed mainly on surface-dwelling fish, taken from shallow dive.	Summer visitor to all Irish coasts. Winters in small numbers in Galway Bay and Strangford Lough.
Hooded crow (*Corvus cornix*)	Diet includes seeds, insects, carrion, young birds and eggs. In coastal areas, will take crabs, **bivalve** and gastropod molluscs.	Common resident throughout Ireland.
Raven (*Corvus corax*)	May feed on practically anything, but mostly on insects and their larvae, worms and other subterranean invertebrates, also berries, grain, small mammals and birds.	Widespread resident throughout Ireland, especially in upland areas.
Brent goose (*Branta bernicla*)	During the winter, it feeds mostly on eel-grass.	Winter migrant from high-Arctic Canada. This population winters almost entirely in Ireland, with small numbers in parts of Britain and France.
Little egret (*Egretta garzetta*)	Takes a wide variety of animals including small fish, frogs, snails and insects	Resident along coasts and rivers throughout Ireland, but still scarce in the Midlands and the north-west.
Grey heron (*Ardea cinerea*)	Diet includes fish, amphibians, small mammals, insects and reptiles.	Common resident at wetlands, estuaries and along rivers throughout Ireland.

CHAPTER 6

Geographical distribution and abundance of significant pathogens associated with *Cerastoderma edule* along the Irish and Welsh coasts

Geographical distribution and abundance of significant pathogens associated with *Cerastoderma edule* along the Irish and Welsh coasts

This manuscript will be submitted for publication with the following author list:

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Abstract

There has been an increase in pathogen emergence in marine ecosystems as a result of a variety of anthropogenic stressors, which in some cases have resulted in changes in the prevalence and geographical distribution of the disease. Improved knowledge of pathogen distribution at different scales and how physical processes and biological effects can influence their spatial patterns is required. The common cockle *Cerastoderma edule* was used as a model group (1) to map the spatial patterns of emerging pathogens - the haplosporidians (obligate protozoan parasites) *Minchinia tapetis* and *Minchinia mercenariae*-like, and *Vibrio* spp. (bacteria) - associated with wild *C. edule* populations at five Irish sites (n=150) and at eight Welsh sites (n=186), some of which are routinely exploited and some of which are not; and (2) to assess the ecological factors that may be shaping the pathogen distribution, diversity, and abundance at a regional and local scale. Newly documented geographic ranges of *Minchinia* and *Vibrio* spp. that are new and emergent in *C. edule* in this region were

reported. No significant differences in the prevalence of these pathogens were found when the two countries were compared. However, significant spatial variability was statistically confirmed between sites, highlighting the importance of site-specific effects in shaping the pathogen spatial distribution. *C. edule* populations exposed to a high concentration of nitrates promoted the presence of *Vibrio* spp. (most likely *V. splendidus*) and 2-pathogen co-occurrence. Low levels of dissolved oxygen may have also facilitated *Vibrio* proliferation, while high temperatures enhanced *M. mercenariae*-like occurrence. Sites with shipping and cockle dredging activity may have also promoted the pathogen prevalence. This study sheds new light on the complex pattern of interactions between the host-pathogen systems and habitat conditions, which will influence the spatial distribution of different pathogen groups in *C. edule*. Findings from this study provide predicted scenarios that can facilitate pathogen emergence and disease development under certain environmental conditions, which are forecasted in the future.

Keywords: *Cerastoderma edule*, *Minchinia*, *Vibrio*, pathogen spatial distribution, environmental stressors, pathogen emergence.

Highlights:

- Newly documented geographic ranges for emergent *Minchinia tapetis* and *Minchinia mercernariae*-like, as well as for *Vibrio* spp., most likely *Vibrio splendidus,* and 2-pathogen co-occurrence in *C. edule* populations were reported.
- Site-specific factors were the main drivers in shaping the pathogen spatial distributions.
- Exposure to a high concentration of nitrates promoted the presence of *Vibrio* spp., most likely *V. splendidus*, as well as the 2-pathogen co-occurrence.
- Seawater temperature was determined as the main driver of *M. mercenariae*-like occurrence and distribution.
- The low concentration of dissolved oxygen may have enhanced *Vibrio* proliferation.
- Shipping and cockle dredging activity may have facilitated the pathogen dispersal and establishment.

6.1. Introduction

Characterising the diversity and distribution of microbial endoparasites and pathogens may be a challenge due to their patchy spatial and temporal distributions and the highly divergent genes that characterise these infectious agents (Hartikainen et al., 2014; Lynch et al., 2020b). Better knowledge of pathogen distributions at different scales and of how physical processes and biological effects can influence their spatial patterns is needed. Understanding some of the current drivers of pathogen emergence and spread has the potential to facilitate the development of early warning systems of pathogen presence and planning tools.

There has been an increase in emerging infectious diseases in marine ecosystems, referring to emergent diseases as the ones that have recently increased in incidence or impact, or have recently moved into a new host population or geographic region (Lederberg et al. 1992, Smolinski et al. 2003). Previous studies have reported changes in the prevalence, geographical distribution and severity of emerging diseases, as a result of a variety of anthropogenic stressors (Harvell et al., 2002; Lafferty et al., 2004; Ward and Lafferty, 2004; Gallana et al., 2013; Sunagawa et al., 2015; Borbor-Cordova et al., 2019; Bramwell et al., 2021). Climate change has been demonstrated as one of the main ecological drivers of emerging infectious diseases (Lafferty, 2009; Bramwell et al., 2021). In northern latitudes, the higher temperatures recorded in the summer months have the potential for increasing the multiplication of microbial pathogens, such as bacteria, while simultaneously stressing hosts, leaving them immunocompromised (Rowley et al., 2014; Zgouridou et al., 2021). Milder winter temperatures may facilitate longer transmission periods and opportunities for reproduction or production of more cohorts of parasites and pathogens (Rowley et al., 2014). Increasing precipitation will reduce salinity in temperate coastal regions, providing new areas for the natural occurrence of pathogenic strains and may also impact the resilience of bivalve hosts (Rowley et al., 2014; Lynch et al., 2020a, b).

Coastal marine environments are subjected to particularly fluctuating environmental conditions and multiple stressors, such as runoff, discharge inputs and climate change, which can have a direct impact on the distribution, life cycle and physiological status of hosts and pathogens inhabiting these environments (Gallana et al., 2013; Yu et al., 2021). There is increasing evidence that a changing environment

will affect the ecology of infectious diseases and the physiology of bivalve species in coastal waters and their resistance to infection (Soon and Zheng, 2020; Zgouridou et al., 2021). Bivalve molluscs, particularly, the well-studied common cockle *Cerastoderma edule*, have proved to be useful bioindicators and model organisms, due to their prominent role in different ecosystem services and widespread distribution (Malham et al., 2012; Longshaw and Malham, 2013; Burdon et al., 2014; Rowley et al., 2014; Carss et al., 2020). As with other filter and deposit-feeding organisms, cockles can accumulate potentially pathogenic agents, such as *Vibrio* and Haplosporidia (Zannella et al., 2017).

Vibrio bacteria are ubiquitous in aquatic environments, including estuaries (Gorrasi et al., 2020), and have been previously associated with *C. edule* (Paillard et al., 2006; Albuixech-Martí et al., 2021; Garcia et al., 2021). The distribution of *Vibrio* is influenced by various environmental parameters, with seawater temperature, salinity and nutrients all being important, and by biological factors (Zhang et al., 2018; Gorrasi et al., 2020). The emergence of pathogenic *Vibrio* species in coastal zones has been predicted based on the unusual increase in seawater temperature due to climate change (Marcogliese, 2008, Vezzulli et al., 2012; Rowley et al., 2014; Gorrasi et al., 2020; Zgouridou et al., 2021). Although *Vibrio* can promptly adapt to environmental changes due to high genome plasticity (King et al., 2019), shifts in *Vibrio* communities in response to fluctuations in environmental parameters have been widely evidenced (Tamplin, 2001; Lin and Schwarz, 2003; Froelich et al., 2012; Johnson et al., 2012; Oliver, 2015). Vezzulli et al. (2016) reported that ocean warming resulted in a significant increase in *Vibrio* abundance and associated human diseases during the 1980s onwards in the North Atlantic.

Poor biogeographical records are available for protistan parasites (Lynch et al., 2020b). 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., 2020b). This has been demonstrated in the case of *Minchinia*, a sparsely recorded haplosporidian genus of five species, with sequence data from molluscs from the US Atlantic coast, Western Australia and Europe (Hartikainen et al., 2014). Up until very

recently, little was known about their diversity and distribution in *C. edule*, however, new reports on *Minchinia tapetis* and *Minchinia mercenariae*-like parasitizing *C. edule* around the coasts of Europe have emerged over the past decade, which have widened their known geographic range (Engelsma et al., 2011; Elliott et al., 2012; Ramilo et al., 2018; Carballal et al., 2020; Lynch et al., 2020b; Albuixech-Martí et al., 2020; Albuixech-Martí et al., 2021). It has been suggested that the dynamics of haplosporidians in their hosts are seasonal and depend on environmental parameters, especially temperature and salinity, which impact the vitality and survival of parasites outside their hosts but also influence host-parasite interactions (Arzul and Carnegie, 2015). Nevertheless, the role of environmental factors in haplosporidian dispersal and ecology is still little understood (Arzul and Carnegie, 2015).

The aims of this study were (1) to map the spatial distribution patterns of three emergent pathogens (*Minchinia tapetis, Minchinia mercenariae*-like and *Vibrio* spp.) and their co-occurrence in *C. edule* from exploited and non-exploited populations along the Irish and Welsh coasts (latitude: from 51°38'04.1"N to 53°47'05.9"N; longitude: from 4°02'27.6"W to 9°58'51.81"W); as well as (2) to assess the environmental and anthropogenic factors that may be influencing pathogen distribution, diversity, and abundance at a regional and a local scale. This study sheds light on the spatial patterns of those pathogens in the current changing environmental context. Findings will provide better knowledge on the pathogen emergence and outbreaks under certain environmental conditions, particularly important among sentinel and bioindicator species such as *C. edule*.

6.2. Materials and Methods

6.2.1. Sampling

Cockles were sampled at five sites on the Irish coast (n=150) and at eight sites on the Welsh coast (n=186) (Table 1) along a latitudinal range (from 51°38'04.1"N to 53°47'05.9"N) and a longitudinal range (from 4°02'27.6"W to 9°58'51.81"W) (Figure 1). Single sampling was done at each site during the period between March 2018 to April 2019. Up to 30 cockles were collected by hand raking from the intertidal area of each site at spring tide (0.5m-1m) (Table 2). All selected sites were bays and estuaries with intertidal sand and mudflats, where a variety of bivalves inhabit, including *C.*

edule, ranging from cockle beds that are commercially harvested to those that are not (Table 1).

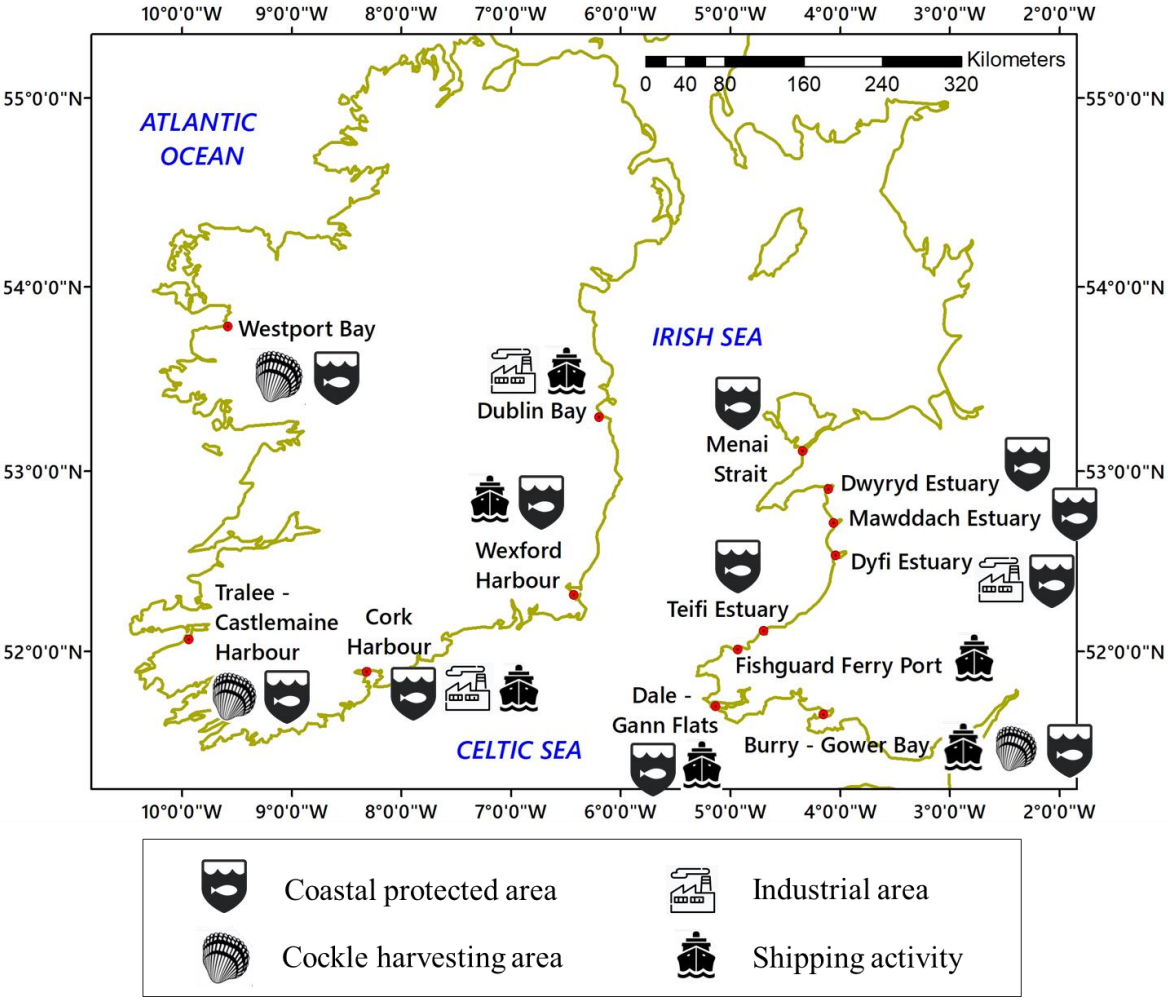

Figure 1: Location of the sample sites along with a representation of the main characteristics of the areas.

Table 1: Sample sites, coordinates, and description, including water quality rating: non-problem area (NP), potential problem area (PPA), problem area (PA) (Eutrophication Status of the OSPAR Maritime Area; 2017); and anthropogenic activities on the areas (Welsh National Marine Plan, Welsh Government, 2020; Shellfish Stocks and Fisheries Review, Marine Institute and BIM, 2018).

	Sites	Coordinates	Eutrophication Status	Description
Welsh Sites	*Menai Strait*	53°8'28.04"N 4°19'43.05"W	NP	Site of Special Scientific Interest (SSSI) and Special Area of Conservation (SAC). A narrow stretch of shallow tidal water, with limited freshwater inputs and a wide range of habitats, such as mudflats and sandflats, reefs, sandbanks, and associated marine communities.
	Dwyryd Estuary	52°53'57.7"N 4°07'00.1"W	NP	Special Area of Conservation (SAC). The estuary, with extensive salt marshes, sand, mud and dune system, is affected by freshwater runoff from the river Dwyryd. The estuary is partially within Snowdonia National Park.
	Mawddach Estuary	52°42'55.5"N 4°02'34.9"W	NP	Site of Special Scientific Interest (SSSI) and Special Area of Conservation (SAC). Wide sandy estuary affected by runoff from the river Mawddach. Extensive intertidal habitats including salt marshes, mud and sand. The estuary is within Snowdonia National Park.
	Dyfi Estuary	52°32'19.1"N 4°02'27.6"W	NP	National Nature Reserve. The Dyfi Estuary has vast areas of mudflats, sandbanks and saltmarsh that are affected by runoff from the river Dyfi. The estuary is partially within Snowdonia National Park. Dyfi has a rich industrial heritage, which has challenges in relation to water quality.
	Teifi Estuary	52°06'15.7"N 4°41'06.8"W	NP	Teifi Estuary, with mudflats and sandbanks, is affected by runoff from the river Teifi. The Teifi Marshes is one of the most important wetland sites in Wales, providing feeding and roosting areas for wetland birds. The estuary is partially within Pembrokeshire Coast National Park.
	Fishguard Ferry Port	52°00'11.6"N 4°59'28.9"W	NP	Fishguard Harbour is a large ferry port and has shipping activity in the bay. It is located adjacent to Pembrokeshire Coast National Park.
	Dale – Gann Flats	51°42'47.7"N 5°10'05.2"W	PPA	Located within Pembrokeshire Coast National Park. The Gann Estuary, near the village of Dale, is a relatively sheltered inlet at the entrance of the Milford Haven Waterway, the most active shipping port in Wales. The Gann's intertidal mudflats and sandflats, 'the flats', are a complex mix of habitats encompassing the largest single area of littoral mixed sediment sheltered muddy gravel habitat in Wales.
	Burry – Gower Bay	51°38'04.1"N 4°09'16.3"W	PA	Gower Peninsula is the UK's first Area of Outstanding Beauty (AONB). The sample site is in the north, where the coast is low-lying with extensive salt marshes and dune systems. The north of the peninsula is home to the cockle-beds of Penclawdd, which are fished commercially. The site is encompassed by Burry Inlet, an enclosed system, designated as a Special Protection Area (SPA) under the E.U. Birds Directive. Shipping activity in Burry Port, at the entrance of the bay.

	Sites	Coordinates	Eutrophication Status	Description
Irish Sites	*Dublin Bay*	53°19'6.79"N 6°11'54.06"W	PPA	The bay is rather shallow with many sandbanks and rocky outcrops. The bay has been subject to pollution from the inflowing watercourses, shipping and port activity, the main water treatment plant for Dublin and sewage discharges at other points.
	Wexford Harbour	52°18'14.01"N 6°23'55.61"W	PPA	Wexford Harbour is the lowermost part of the estuary of the River Slaney. Shallow marine water is a principal habitat but, at low tide, extensive areas of intertidal flats are exposed. The site is a Special Protection Area (SPA) under the E.U. Birds Directive. Fishing port and leisure shipping activity in the area.
	Cork Harbour – Cuskinny	51°51'29.94"N 8°15'49.50"W	PPA	A large, sheltered bay system, with several river estuaries and intertidal mudflats. Intense shipping and industrial activity in the harbour. Cuskinny, within the harbour, is a Marsh Nature Reserve supporting an array of habitats, within a relatively small area, for a diverse community of wildlife.
	Tralee – Castlemaine Harbour	52° 8'37.50"N 9°58'51.81"W	NP	A sandy beach bordered by two streams. The intertidal sediments vary from muddy sands on the upper shore to firm rippled sands on the lower, more exposed shore. There is a small-scale commercial hand-gathering cockle fishery in Castlemaine Harbour. The site is a Special Protection Area (SPA) under the E.U. Birds Directive.
	Westport Bay	53°47'05.9"N 9°39'09.1"W	NP	Westport Bay is a shallow inlet, approached by narrow and intricate channels between a number of islets in Clew Bay. Commercial cockle (*C. edule*) stock occurs in Clew Bay, but this stock has not been commercially fished in recent years. The site is a Special Area of Conservation (SAC).

Table 2: Collection date and sample size at each site in Ireland and Wales; along with the environmental data: monthly mean of seawater temperature (Temp), salinity (Sal), dissolved oxygen (O_2), pH, and concentration of nitrate (NO_3) corresponding to the collection date.

	Sites	Collection date	Sample size	Temp (°C)	Sal (PSU)	O_2 (mmol/m^3)	pH	NO_3 (mmol/m^3)
Irish Sites	*Dublin Bay*	April 2019	30	9.82	33.77	2991.00	8.09	0.40
	Wexford Harbour	April 2019	30	10.20	34.31	2950.00	8.09	0.40
	Cork Harbour – Cuskinny	March 2019	30	9.35	34.64	2938.00	8.06	4.90
	Tralee – Castlemaine Harbour	March 2019	30	8.66	30.43	3106.00	8.04	**16.10**
	Westport Bay	March 2019	30	7.93	30.67	3161.00	8.03	13.40
Welsh Sites	*Menai Strait*	March 2018	22	6.52	34.54	3052.00	**8.10**	0.00
	Dwyryd Estuary	June 2018	10	**17.61**	30.44	2740.00	7.95	3.50
	Mawddach Estuary	June 2018	28	17.60	31.13	2799.00	7.97	2.20
	Dyfi Estuary	May 2018	15	12.43	32.33	**3298.00**	8.05	4.60
		Nov. 2018	14	10.21	33.32	2820.00	8.04	8.30
	Teifi Estuary	June 2018	21	13.89	34.07	2836.00	7.98	0.20
	Fishguard Ferry Port	June 2018	26	12.61	34.40	2877.00	8.00	0.00
	Dale - Gann Flats	Sep. 2018	27	16.71	**35.08**	2502.00	8.00	0.10
	Burry - Gower Bay	June 2018	23	16.79	30.61	2781.00	8.00	0.10

The highest environmental values are in bold font.

6.2.2. Sample processing and diagnostic methods

6.2.2.1. Molecular techniques for pathological screening:

A small piece of gill tissue (2-5mm^2 of tissue) from each cockle of the Irish sites was taken and stored in 70% ethanol for subsequent pathological screening. Genomic DNA from the cockle gill tissue was extracted using the chelex-100 extraction method (Walsh et al., 1991; Lynch et al., 2008). Tissue cross-sections, including digestive gland, mantle, gill, and adductor muscle, from each cockle of the Welsh sites, were dissected and homogenised for DNA extraction using a CTAB and phenol-chloroform procedure (Winnepenninckx et al., 1993).

Standard PCR screening for *Minchinia* species detection was carried out in all DNA extractions using specific primers for *Minchinia tapetis* (TAP-For/Rev) and *Minchinia mercenariae*-like (MER-For/Rev) (Albuixech-Martí et al., 2020; Table 3).

Two positive controls (purified DNA of the respective *Minchinia* species) were included in each PCR assay to confirm that the amplification worked. Likewise, negative controls (two replicates with ddH_2O) were included in each PCR assay to confirm the absence of contamination. Amplification was conducted following the reaction mixture and thermocycling conditions, as well as the visualisation of the product, described in Albuixech-Martí et al. (2020).

Standard PCR screening for *Vibrio* spp. in all DNA extractions was conducted using generic primers Vib-F3/R3, developed in our lab for the detection of the *Vibrio* genus (Kett et al., 2022) (Table 3). A total of 2 µl of DNA per individual was used in a total volume of 27.5µl of the reaction mixture containing: 5µl of 5x green buffer, 5µl of dNTPs (1.25mM), 0.5µl of $MgCl_2$, 0.25µl of forward and reverse primers, 0.1µl of GoTaq polymerases, 1.5µl of DMSO and 12.9µl of ddH_2O. Cycling conditions consisted of an initial denaturation of the sample at 95°C for 1 minute followed by 35 cycles of 94°C for 20s, 56°C for 30s, 72°C for 30s and a final elongation at 72°C for 7 minutes. Purified DNA of the pathogen was used as a positive control. Two positive controls were included in each PCR assay to confirm that the amplification worked. Likewise, negative controls (two replicates with ddH_2O) were included in each PCR assay to confirm the absence of contamination. Electrophoresis of the amplification products was conducted in a 2% agarose gel. The expected amplified product (amplicon) size for *Vibrio* spp. was 286bp.

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

	Primer sequence (5'–3')			
Primer pair	**Forward**	**Reverse**	**Product size (bp)**	**Primer specificity**
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
Vib-F3 Vib-R3	CAA CAG AAG AAG CAC CGG CT	CAC GCT TTC GCA TCT GAG TG	286	*Vibrio* spp.

Quantitative PCR (qPCR) for detection and quantification of *Vibrio aestuarianus* in the cockle samples from the Irish sites that were positive for *Vibrio* spp. (n=50) was performed using specific primers (Table 4) for detection of the molecular chaperone *dnaJ* gene, following the protocol of McCleary and Henshilwood (2015). Negative template controls consisting of 5 µl of double-distilled water (ddH_2O) were included in triplicates in each run. 5 µl of *V. aestuarianus* DNA suspension was used in triplicates as positive controls in each run. All samples were performed in triplicates using 5 µl of DNA. C_t (Cycle threshold) values were used to determine PCR quantification and detection limits by Thermo Hybaid PCR express thermal cycler. A tested sample was considered positive if its mean C_t value was below 37. Six prepared 1:10 serially diluted *V. aestuarianus* DNA standards were tested in triplicates as stated in McCleary and Henshilwood (2015). Standard curves aim at checking the efficacy of the PCR reaction and at estimating the number of bacterial DNA copies present in tested samples. Results are interpreted by linear regression for slope analysis, and the PCR amplification efficiency (E), calculated according to: $E = [10^{1/(-slope)}] - 1$, should range from 95% to 105% (McCleary and Henshilwood, 2015). Absolute quantitation for DNA copies is carried out by comparing the C_t obtained against the standard curve with known copy numbers. In the case of the Welsh samples, there was not enough quantity of sample to conduct the qPCR analysis.

Table 4: Description of qPCR primer pair and probe.

Primers	**Sequence**	**Specificity**
dnaJ f420	GTGAAGGGACGGGTGCTAAG	
dnaJ r456	CCATGACAAGTGCCACAAGTCT	*Vibrio aestuarianus*
dnaJ p441 (probe)	FAM-AGGGCACGTCGGC-MGB	

Direct sequencing was carried out on representative PCR products (n=48) amplified using generic *Vibrio* Vib-F3/R3 primers (Kett et al., 2022) to identify the *Vibrio* species present in the samples from Irish and Welsh sites. Genomic DNA from selected individuals was isolated and purified using the QIAquick Gel Extraction Kit (QIAgen) prior to direct sequencing commercially (Source Bioscience). Each sequence was matched against the National Center for Biotechnology Information

(NCBI) nucleotide database with BLASTn (Basic Local Alignment Search Tool), which finds regions of local similarity between sequences to identify and confirm the DNA being detected in the PCRs.

6.2.2.2. Histology:

A transverse section, including visceral mass, gill, mantle and foot tissues, of each cockle collected at the Irish sites, was placed in an individually labelled histocassette and was preserved in Davidson's fixative for 24 hours prior to processing. Paraffin-embedded tissue blocks were sectioned at 5µm and stained using Harris' haematoxylin and eosin (Howard and Smith, 1983). Tissue sections were screened at a magnification of 40x and under oil at 100x for visualization of the pathogens and tissue pathology. The histological examination was used as a validation method of the results from the molecular analysis.

6.2.3. Environmental data

This study was conducted using E.U. Copernicus Marine Service Information (marine.copernicus.eu/services-portfolio/access-to-products). Five environmental parameters critical to water quality in estuarine and marine environments were selected for this study: seawater temperature, salinity, dissolved oxygen, pH, and concentration of nitrate in seawater. Recorded data in temperature (°C), salinity (PSU), dissolved oxygen (mmol/m^3), pH, and concentration of nitrate in seawater (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) for the corresponding collection dates at each sample site (coordinate-based) along the Irish and Welsh coasts (Table 2).

6.2.4. Statistical analysis

Statistical analysis was performed in R version 3.2.3. statistical software. The pathogen occurrence was modelled as the presence/absence of pathogen DNA detected by PCR in each individual cockle for each pathogen group and for the pathogen co-occurrence, used as the response variables in the subsequent analysis to

examine the spatial variability and the effects of the environmental variables on the pathogen spatial distribution.

Chi-square tests (χ^2) between the observed values and the expected values of the prevalence of each combination of pathogen co-occurrence were conducted to assess whether the total observed prevalence values were significantly different ($p(\chi^2) < 0.05$) from the expected values or were consistent with random chance ($p(\chi^2) > 0.05$).

In order to determine whether, overall, the PCR-positive cases of the single pathogen detection, 2-pathogen co-occurrence and uninfected individuals differed between the Irish sites and the Welsh sites, the mean percentages of PCR-positive cases of each pathogen, 2-pathogen co-occurrence and of uninfected individuals, after grouping all the samples, were calculated for each coast. Differences between the mean values at the Irish sites and at the Welsh sites were then analysed with an unpaired 2-sample t-test when the distribution followed by the corresponding PCR-positive/uninfected individuals was normal. If the PCR-positive/uninfected individuals followed a non-normal distribution, the significance was assessed by the Mann-Whitney U-test which examines the difference between the medians of two unmatched samples. Significance was established at p-value < 0.05.

Fisher's Exact Tests were conducted to determine whether the prevalence observed for each pathogen and 2-pathogen co-occurrence, as well as the frequency of uninfected individuals, was significantly different ($p(Fisher) < 0.05$) among the sample sites. This test was used instead of Pearson's Chi-squared tests to gain accuracy since the frequencies of the pathogen occurrences in some of the sites were < 4.

A series of Generalised Linear Mixed Models (GLMMs), following binomial distributions for presence/absence data (lme4 package; Bates et al., 2015) (considering the presence/absence data for each pathogen species; and, subsequently, the pathogen co-occurrence presence/absence data), were conducted to investigate the relative importance of the environmental factors (explanatory variables) in determining the presence of each pathogen and, subsequently, the pathogen co-occurrence in the field. Each GLMM included pathogen presence/absence as the response variable, none (Null_Model) or a single explanatory variable (1-factor models) as the fixed effect and collection date as a random term (in order to control for the variation due to the

different sampling time). Each 1-factor model was compared to the Null_Model (intercept-only model) using the likelihood ratio test following Chi-square distributions to determine whether the presence of pathogens observed was significantly ($p(\chi^2)<0.05$) affected by the environmental factors tested. All the explanatory variables were standardized (x-mean/SD) to improve comparisons in the models. Visual inspection of residual plots and model validation with each above model revealed that the model assumptions were satisfied in all the cases.

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 explanatory 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. Model comparison (bbmle package, Bolker et al., 2009) was done for the series of 1-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 ΔAIC values ≤ 2 (i.e., not statistically different from the best performing model with ΔAIC = 0) (Thomas, 2017).

6.3. Results

In total, 336 cockles were screened to determine the prevalence of each pathogen group and pathogen co-occurrence in *C. edule* at the Irish (n=150) and Welsh (n=186) sites (Table 5). Single pathogen detection was considered when only one pathogen group was detected in *C. edule* individuals, while 2-pathogen co-occurrence was described as the individuals with two pathogen groups simultaneously present. The 2-pathogen co-occurrence included the co-occurrence of both *Minchinia* species or either one *Minchinia* species and *Vibrio* spp. Due to the low number (2 individuals in Tralee - Castlemaine Harbour) of pathogen co-occurrence involving the three pathogens, this co-occurrence was not considered separately for statistical and

spatial analysis purposes, instead, these two individuals were included as 2-pathogen co-occurrence. The spatial patterns of each pathogen group (considering the single occurrence and the co-occurrence) and, subsequently, the 2-pathogen co-occurrence were assessed at a regional and a local scale.

Prior to the spatial analysis at a regional and a local scale, the observed total prevalence for each combination of co-occurrence of pathogens (Table 5) were compared to the expected values in order to assess if the co-occurrence observed values were consistent with random chance. Being 20.2% (n=68/336) the total prevalence of *Vibrio* spp. and assuming no bias towards coinfection, it would be reasonable to expect 2 of 12 (0.20 × 12) animals infected with *M. tapetis* to be coinfected with *Vibrio*. However, the observed prevalence of coinfection with *Vibrio* spp. and *M. tapetis* (1.8%, n=6/336) was significantly higher ($p(\chi^2) < 0.05$) than the prevalence expected by random chance (0.6%, n=2/336). The observed prevalence of coinfection with *Vibrio* spp. and *M. mercenariae*-like (10.7%, n=36/336) was also significantly higher ($p(\chi^2) < 0.05$) when it was compared to the prevalence expected by random chance, i.e., 9 of 44 (0.20 × 44) animals infected with *M. mercenariae*-like would be expected to be coinfected with *Vibrio* (2.7%, n=9/336). Regarding the coinfection with both *Minchinia* species, being 3.6% (n=12/336) the total prevalence of *M. tapetis*, the observed value of coinfection with *M. mercenariae*-like (1.5%, n=5/336) was also significantly higher ($p(\chi^2) < 0.05$) than expected by random chance, i.e., 2 of 44 (0.04 × 44) animals infected with *M. mercenariae*-like would be expected to be coinfected with *M. tapetis* (0.6%, n=2/336). However, the one out of five (0.20 x 5) (0.3%, n=1/336) individuals that was expected to be coinfected with both *Minchinia* species and also *Vibrio* spp. was consistent ($p(\chi^2) > 0.05$) with the observed prevalence of triple infection at 0.6% (n=2/336).

6.3.1. Diversity and prevalence of single pathogen detection and 2-pathogen co-occurrence at a regional scale

The distribution of each pathogen group and, subsequently, the pathogen co-occurrence was compared between Ireland and Wales to determine any significant difference at a regional scale.

No significant differences (p-value > 0.05) in *Minchinia* species prevalence were revealed between Ireland and Wales. At the Irish sites, on average, 5.3 [2.3, 10.2]% (mean [95% Confidence Interval (CI)]) of the cockles showed presence of *Minchinia tapetis* exclusively, while 23.3 [16.8, 30.9]% displayed presence of *Minchinia mercenariae*-like (Figure 2). The mean prevalence of single detection of *M. tapetis* (2.4 [0.6, 5.4]%) and, alternatively, *M. mercenariae*-like (6.9 [2.2, 9.0]%) were lower at the Welsh sites (Figure 2). Histological sections (n = 78 screened) revealed cells that fit the morphology of haplosporidian cells despite the deterioration of tissue integrity in those samples. In those PCR-positive *C. edule* samples, small haplosporidian cells of the expected size ranging from 3 to 5 µm in diameter (mean = 4.5 µm) (Ramilo et al., 2018) were visualised consistent with either *M. tapetis* or *M. mercenariae*-like, along with multinucleate plasmodia that can range from 6 to 15 µm in diameter (mean = 8.5 µm) and enclose from 3 to 14 nuclei (Ramilo et al., 2018; de Montaudouin et al., 2021) (Figure 3).

No significant difference (p-value > 0.05) in *Vibrio* prevalence was revealed between Ireland and Wales. Single detection of *Vibrio* spp., on average, was higher in *C. edule* at the Welsh sites (mean [95% CI]; 24.4 [19.7, 32.7]%) than at the Irish sites (13.3 [8.3, 19.8]%) (Figure 2). No samples that were positive for *Vibrio* were confirmed by qPCR to be *Vibrio aestuarianus*. Direct sequencing was carried out on representative PCR products from Irish (n=24) and Welsh (n=24) sample sites to identify the *Vibrio* species present in the samples. Using BLASTn, the sequenced samples from both coasts showed a query length of 63 to 255bp with a 60 to 90% query coverage and 91.5 to 100% percent identity to *Vibrio splendidus* deposited by Gao (2020) (MT445179.1; MT445177.1) and by Landreau et al. (2020) (MT345091.1). One sample from Dwyryd Estuary, with a query length of 80bp, had 73% query coverage and 95.1% percent identity to *Vibrio mediterranei* deposited by Jiang (2011) (HE584789.1) and 67% query coverage and 96.4% percent identity to *V. aestuarianus* deposited by Garcia (2018) (MK307696.1). Four samples (from Cork Harbour, Tralee, and Fishguard Ferry Point), with a query length of 82 to 214bp, showed 93.75 to 97.73% percent identity to *V. splendidus* deposited by Gao (2020) (MT445179.1; MT445177.1) and by Landreau et al. (2020) (MT345091.1). However, these four samples were identified as *Vibrio* spp., as the query coverage was too short (44-55%) to differentiate robustly between species. Given the general consistency of

the sequencing results, the positive PCR cases were defined as *Vibrio* spp. most likely *V. splendidus* for statistical and analysis purposes.

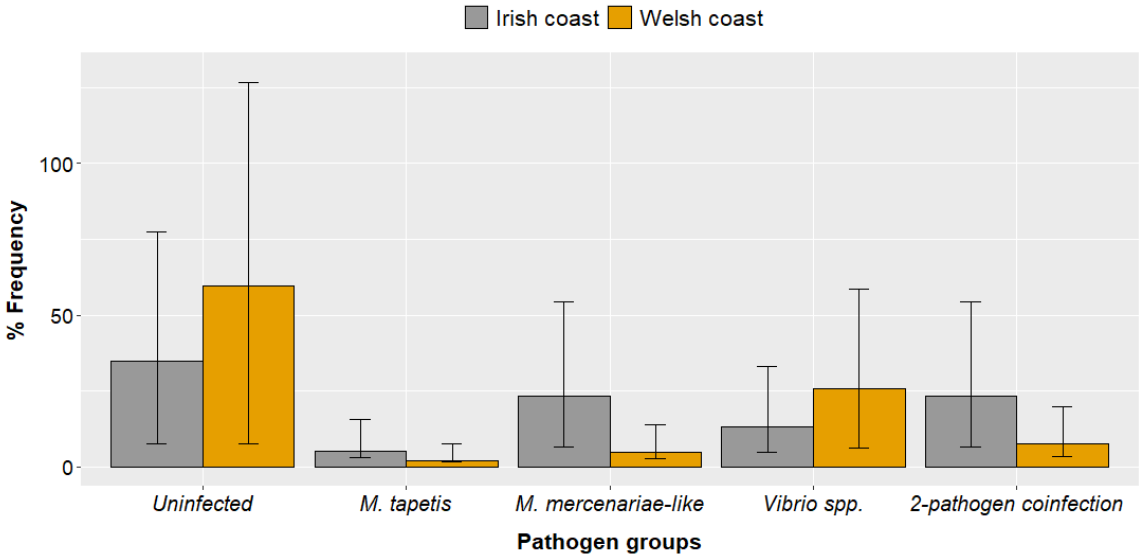

Figure 2: Mean frequency (%) ± Binomial Confidence Intervals (95% CI) of uninfected individuals, single detection of *Minchinia tapetis*, *M. mercenariae*-like, and *Vibrio* spp., most likely *V. splendidus*, and 2-pathogen co-occurrence in *Cerastoderma edule* at Irish and Welsh coasts.

The mean prevalence of co-occurrence of *Minchinia* and *Vibrio* species in *C. edule* was also lower at the Welsh sites (mean [95% CI]; 8.7 [4.2, 12.3]%; co-occurrence of one *Minchinia* species and *Vibrio* spp.) than at the Irish sites (23.3 [16.8, 30.9]%; including co-occurrence of both *Minchinia* species or either one *Minchinia* species and *Vibrio* spp.) (Figure 2). Nevertheless, no significant differences (p-value > 0.05) in the prevalence of 2-pathogen co-occurrence were revealed between Ireland and Wales. Histological examination of the individuals with pathogen co-occurrence revealed uninucleate haplosporidian cells consistent with either *M. tapetis* or *M. mercenariae*-like in addition to extracellular bacterial colonies (Figure 3).

A higher mean frequency of uninfected *C. edule* was recorded at the Welsh sites (mean [95% CI]; 57.7 [52.3, 66.8]%) compared to the Irish sites (34.7 [27.1, 42.9]%) (Figure 2). Nevertheless, no significant differences (p-value > 0.05) were revealed between the mean frequency of uninfected individuals between Ireland and Wales.

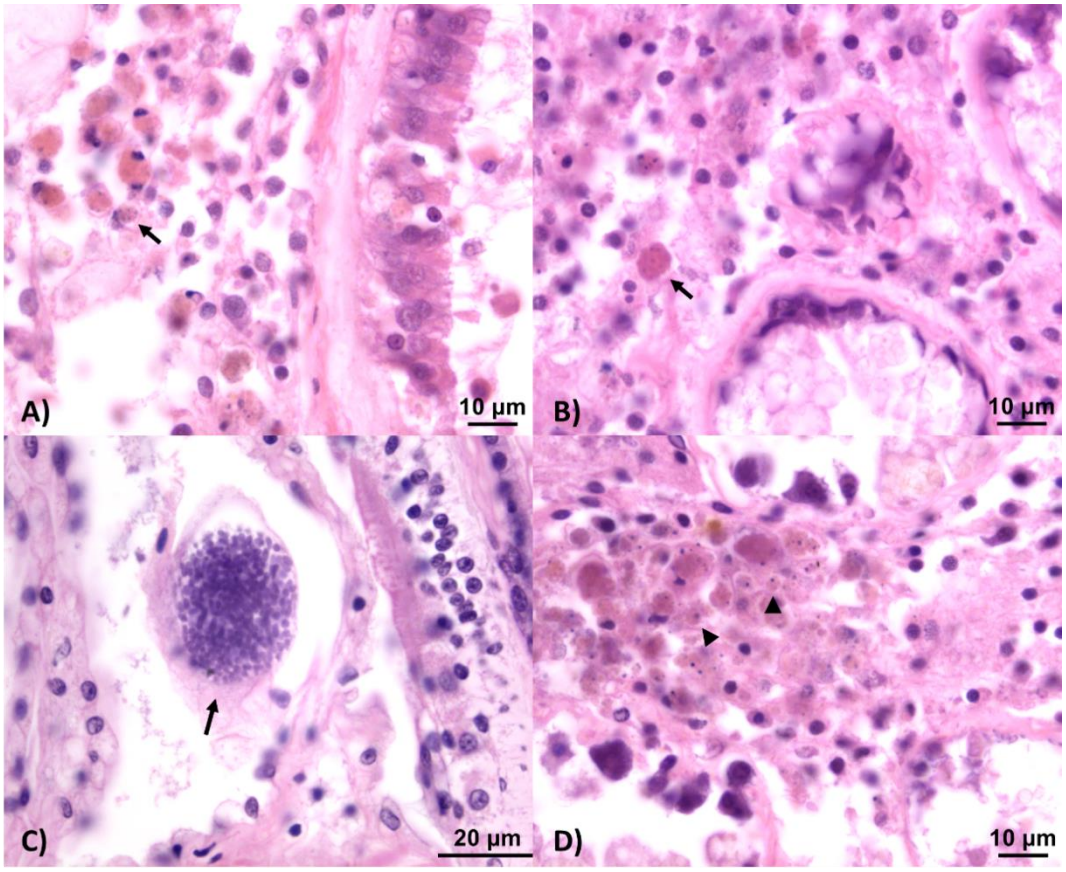

Figure 3: A) and B) Multinucleate plasmodia (black arrow) in the connective tissue of *Cerastoderma edule* from the Irish coast; C) extracellular bacterial colony (black arrow) in the connective tissue of *C. edule* from the Irish coast; D) uninucleate haplosporidian cells (head arrow) in the connective tissue of the digestive system in *C. edule* from the Irish coast.

6.3.2. Spatial variability in the detection of each pathogen group and the 2-pathogen co-occurrence at a local scale

Since no significant differences were found at a regional scale, the differences in the spatial distribution of each pathogen group and the pathogen co-occurrence between sites were also examined at a local scale.

Significant differences (p(Fisher) < 0.001) in the prevalence of each pathogen group, i.e., *M. tapetis*, *M. mercenariae*-like, and *Vibrio* spp. (*V. splendidus*), was displayed between the sample sites. *M. tapetis* was not detected in *C. edule* at most of the Welsh sites, except at Teifi Bay, with the highest prevalence of *M. tapetis* single detection (19%; n=4/21) among the Irish and Welsh sites (Table 5, Figure 4). *C. edule* at Cork Harbour had the highest prevalence of *M. mercenariae*-like single detection

(46.7%, n=14/30) among the Irish and Welsh sites (Table 5, Figure 4). *M. mercenariae*-like single detection was only observed in *C. edule* at Dwyryd Estuary (30%; n=3/10), at Burry – Gower Bay (21.7%; n=5/23), and at Dyfi Estuary (3.4%; n=1/29) among the Welsh sites (Table 5, Figure 4). Single detection of *Vibrio* spp. (*V. splendidus*) was not observed in *C. edule* at Burry – Gower Bay and Wexford Harbour (Table 5, Figure 4). *C. edule* sampled at Dale – Gann Flats showed the highest prevalence of *Vibrio* spp. (*V. splendidus*) single detection (63%; n=17/27) among the Irish and Welsh sites (Table 5, Figure 4).

Significant spatial variability (p(Fisher) < 0.001) between the sample sites was also detected for 2-pathogen co-occurrence. The highest prevalence of 2-pathogen co-occurrence (including co-occurrence of both *Minchinia* species or either one *Minchinia* species and *Vibrio* spp.) was displayed in *C. edule* at Tralee - Castlemaine Harbour (56.7%, n=17/30) (Table 5, Figure 4). *C. edule* at Tralee - Castlemaine Harbour showed the highest single detection of *Vibrio* spp. (*V. splendidus*) (36.7%, n=11/30) among the Irish sites (Table 5, Figure 4). The only Welsh *C. edule* that had 2-pathogen co-occurrence were observed at Dyfi Estuary (34.5%; n=10/29; with *M. mercenariae*-like and *Vibrio* spp. (*V. splendidus*)), Dwyryd Estuary (30%; n=3/10; with *M. mercenariae*-like and *Vibrio* spp. (*V. splendidus*)) and Teifi Estuary (4.8%; n=1/21; with *M. tapetis* and *Vibrio* spp. (*V. splendidus*)) (Table 5, Figure 4).

Significant differences (p(Fisher) < 0.001) in the frequency of uninfected cockles were also shown between the sample sites. Fishguard Ferry Port *C. edule* exhibited the highest presence of uninfected individuals (88.5%; n=23/26) among the Irish and Welsh sites, with only *Vibrio* spp. (*V. splendidus*) detected (11.5%; n=3/26) (Table 5, Figure 4). Tralee - Castlemaine Harbour, in turn, was the site with the lowest frequency of uninfected individuals (6.7%; n=2/30) (Table 5, Figure 4) among the Irish and Welsh sites.

Table 5: Collection date and prevalence (%, PCR-positive individuals/screened individuals) of single detection of *Minchinia tapetis*, *M. mercenariae*-like, and *Vibrio* spp., most likely *V. splendidus*, and each combination of pathogen co-occurrence, as well as frequency (%, uninfected individuals/screened individuals) of uninfected *Cerastoderma edule* at each Irish and Welsh sample site.

	Sites	Collection date	*M. tapetis*	*M. mercenariae*-like	*Vibrio* spp.	*M. tapetis* and *Vibrio* spp.	*M. mercenariae*-like and *Vibrio* spp.	*M. tapetis* and *M. mercenariae*-like	*M. tapetis*, *M. mercenariae*-like and *Vibrio* spp.	Uninfected
Irish Sites	*Dublin Bay*	April 2019	13.3% (4/30)	20% (6/30)	13.3% (4/30)	3.3% (1/30)	23.3% (7/30)	**6.7% (2/30)**	0% (0/30)	20% (6/30)
	Wexford Harbour	April 2019	6.7% (2/30)	43.3% (13/30)	0% (0/30)	0% (0/30)	3.3% (1/30)	**6.7% (2/30)**	0% (0/30)	40% (12/30)
	Cork Harbour – Cuskinny	March 2019	6.7% (2/30)	**46.7% (14/30)**	10% (3/30)	0% (0/30)	6.7% (2/30)	0% (0/30)	0% (0/30)	30% (9/30)
	Tralee – Castlemaine Harbour	March 2019	0% (0/30)	0% (0/30)	36.7% (11/30)	**6.7% (2/30)**	**43.3% (13/30)**	0% (0/30)	**6.7% (2/30)**	6.7% (2/30)
	Westport Bay	March 2019	0% (0/30)	6.7% (2/30)	6.7% (2/30)	**6.7% (2/30)**	0% (0/30)	3.3% (1/30)	0% (0/30)	76.7% (23/30)
	Totals		5.3% (8/150)	23.3% (35/150)	13.3% (20/150)	3.3% (5/150)	15.3 % (23/150)	3.3% (5/150)	1.3% (2/150)	34.7% (52/150)
Welsh Sites	*Menai Strait*	March 2018	0% (0/22)	0% (0/22)	36.4% (8/22)	0% (0/22)	0% (0/22)	0% (0/22)	0% (0/22)	63.6% (14/22)
	Dwyryd Estuary	June 2018	0% (0/10)	30% (3/10)	20% (2/10)	0% (0/10)	30% (3/10)	0% (0/10)	0% (0/10)	20% (2/10)
	Mawddach Estuary	June 2018	0% (0/28)	0% (0/28)	21.4% (6/28)	0% (0/28)	0% (0/28)	0% (0/28)	0% (0/28)	78.6% (22/28)
	Dyfi Estuary	May 2018	0% (0/15)	6.7% (1/15)	20% (3/15)	0% (0/15)	33.3% (5/15)	0% (0/15)	0% (0/15)	40% (6/15)
		Nov. 2018	0% (0/14)	0% (0/14)	57.1% (8/14)	0% (0/14)	35.7% (5/14)	0% (0/14)	0% (0/14)	7.1% (1/14)
	Teifi Estuary	June 2018	**19% (4/21)**	0% (0/21)	4.8% (1/21)	4.8% (1/21)	0% (0/21)	0% (0/21)	0% (0/21)	71.4% (15/21)
	Fishguard Ferry Port	June 2018	0% (0/26)	0% (0/26)	11.5% (3/26)	0% (0/26)	0% (0/26)	0% (0/26)	0% (0/26)	**88.5% (23/26)**
	Dale – Gann Flats	Sep. 2018	0% (0/27)	0% (0/27)	**63% (17/27)**	0% (0/27)	0% (0/27)	0% (0/27)	0% (0/27)	37% (10/27)
	Burry – Gower Bay	June 2018	0% (0/23)	21.7% (5/23)	0% (0/23)	0% (0/23)	0% (0/23)	0% (0/23)	0% (0/23)	78.3% (18/23)
	Totals		2.2% (4/186)	4.8% (9/186)	25.8% (48/186)	0.5% (1/186)	7.0% (13/186)	0% (0/186)	0% (0/186)	59.7% (111/186)

The highest prevalence (%) of each pathogen and each combination of pathogen co-occurrence, and the highest frequency (%) of uninfected individuals at each site are in bold.

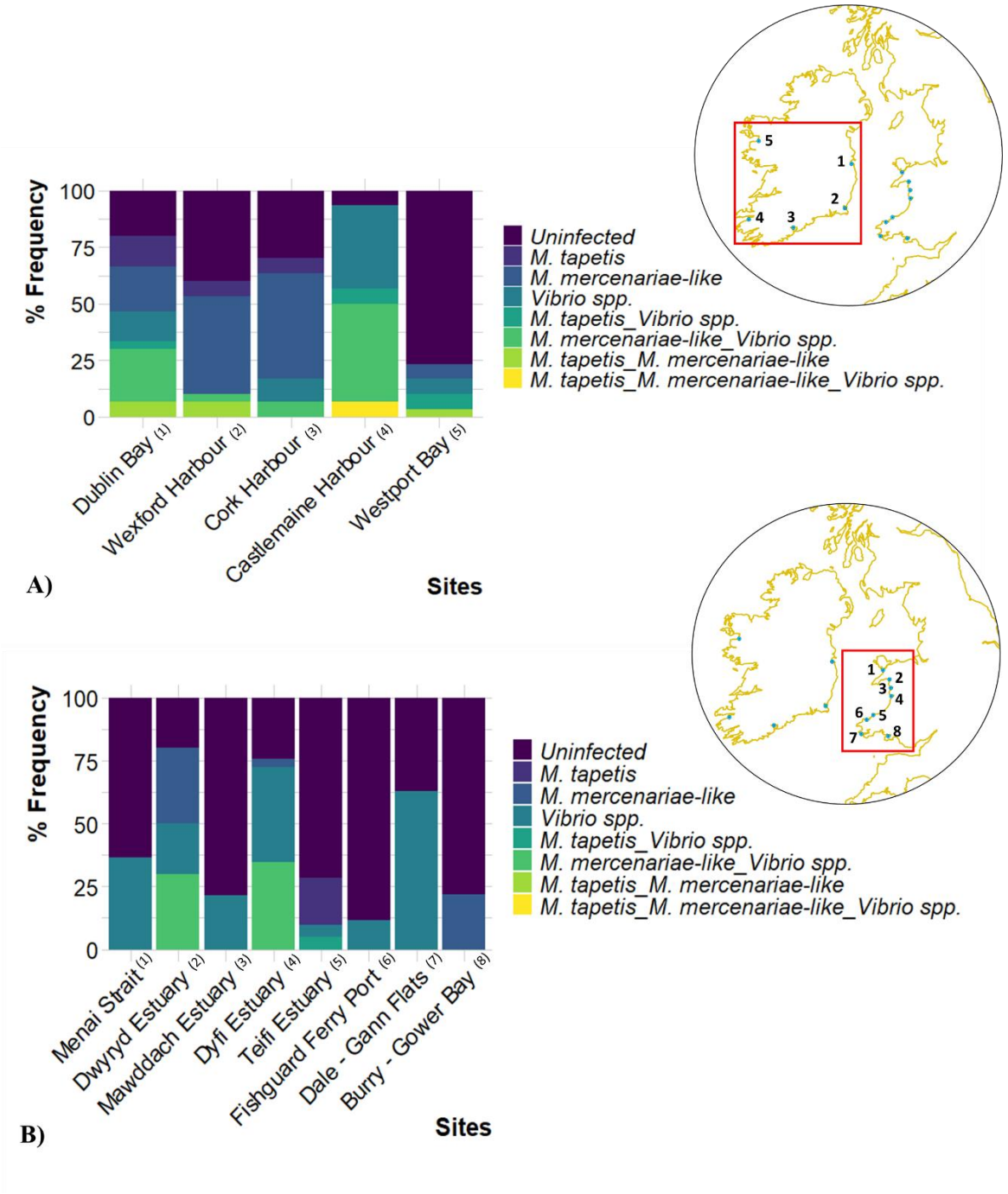

Figure 4: A) Sample site locations in Ireland; frequency (%) of uninfected individuals, single detection of *Minchinia tapetis*, *M. mercenariae*-like, and *Vibrio* spp., most likely *V. splendidus*, and each combination of pathogen co-occurrence in *Cerastoderma edule* at each Irish site. B) Sample site locations in Wales; frequency (%) of uninfected individuals, single detection of *M. tapetis*, *M. mercenariae*-like, and *Vibrio* spp., most likely *V. splendidus*, and each combination of pathogen co-occurrence in *C. edule* at each Welsh site.

6.3.3. Effects of environmental factors on each pathogen group and the 2-pathogen co-occurrence distributions at a local scale

The significant differences between sample sites were subsequently examined in relation to site-specific environmental factors to determine the drivers that may be shaping the pathogen distribution at a local scale.

None of the environmental variables (Table 2) came out as significant factors in determining *M. tapetis* distribution, since the 1-factor models showed no significant differences with the null model (intercept-only model; AIC=176.0; ΔAIC≤2; Supplementary material Table S1).

Among the 1-factor models, seawater temperature was the best performing driver shaping the *M. mercenariae*-like distribution in the study (with the lowest AIC=329.8; Supplementary material Table S2). Higher *M. mercenariae*-like occurrence was observed at sites where the collection took place in June 2018 (Welsh sites) and March and April 2019 (Irish sites) (Figure 5). The other 1-factor models were statistically less plausible than the best performing model (ΔAIC≥2; Supplementary material Table S2).

Based on the models, the concentration of nitrate in seawater was the best performing driver associated with *Vibrio* distribution (with the lowest AIC=365.5; Supplementary material Table S3). A higher prevalence of *Vibrio* spp. (*V. splendidus*) was observed at the sites with higher concentrations of nitrate in the seawater, such as Tralee - Castlemaine Harbour and Dyfi Estuary (Figure 5). Neither of the other environmental factors came out as significant factors in determining *Vibrio* distribution (statistically less plausible than the best performing model, ΔAIC≥2; Supplementary material Table S3).

Among the 1-factor models, the concentration of nitrate was the best performing driver (with the lowest AIC=239.7) associated with 2-pathogen co-occurrence distribution (including co-occurrence of both *Minchinia* species or either one *Minchinia* species and *Vibrio* spp.) (Supplementary material Table S4). A higher 2-pathogen co-occurrence was observed at the sites with higher concentrations of nitrate in seawater, such as Tralee - Castlemaine Harbour and Dyfi Estuary (Figure 5). The rest of the 1-factor models were not significant in determining the 2-pathogen co-

occurrence distribution (statistically less plausible than the best performing model, ΔAIC≥2; Supplementary material Table S4).

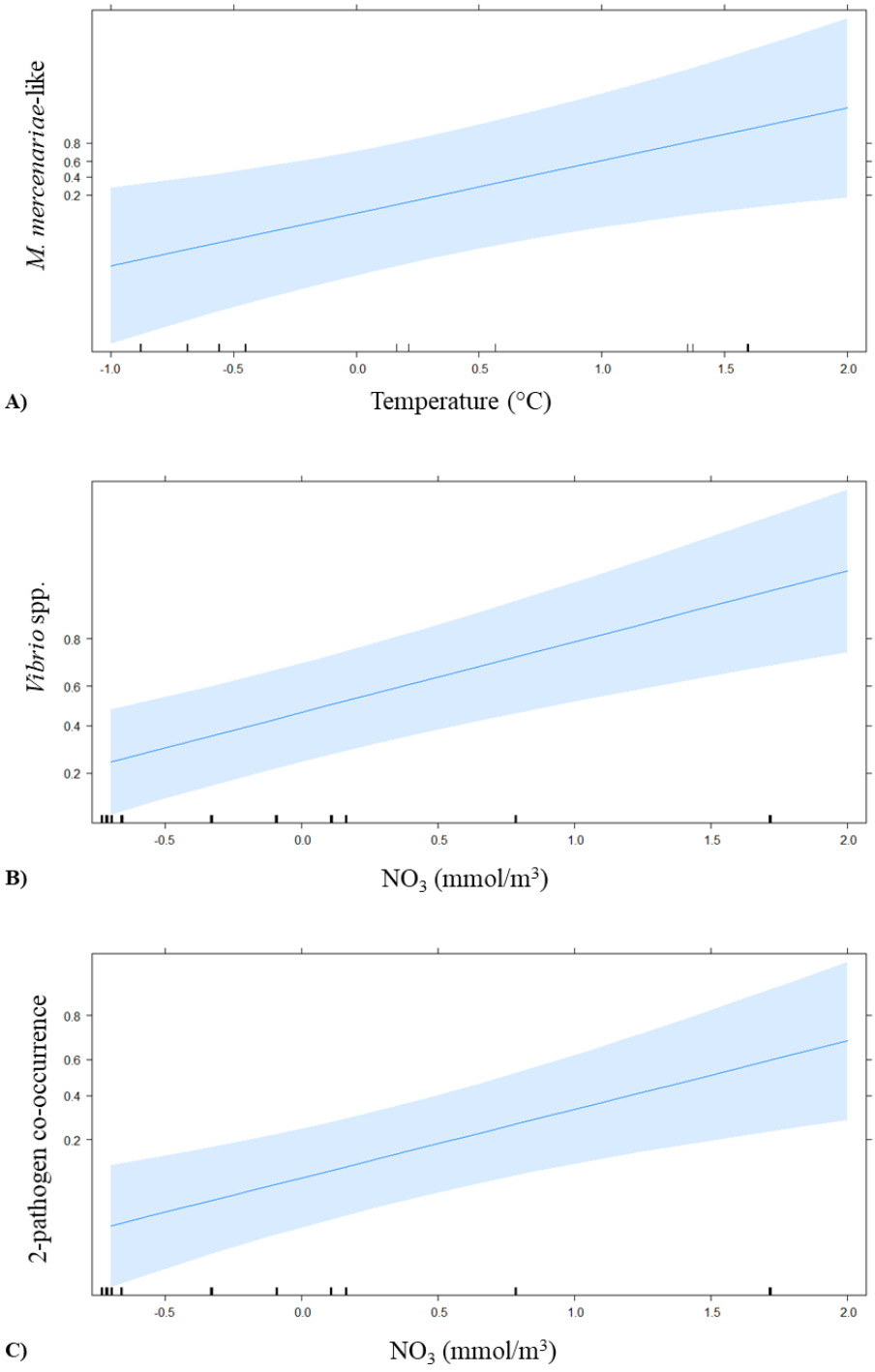

Figure 5: Plots of the effects, based on the 1-factor models, of A) the temperature (°C) on *Minchinia mercenariae*-like distribution; B) the concentration of nitrates (mmol/m^3) on *Vibrio* spp., most likely *V. splendidus,* distribution; and C) the concentration of nitrates (mmol/m^3) on 2-pathogen co-occurrence distribution (including co-occurrence of both *Minchinia* species or either one *Minchinia* species and *Vibrio* spp.) in *Cerastoderma edule*.

6.4. Discussion

The spatial distribution of *Minchinia* and *Vibrio* spp. in *C. edule* at newly documented latitudinal and longitudinal ranges along the Irish and Welsh coasts was reported for the first time in this collaborative study. This study examined the pathogen distributions at a regional and a local scale, and the findings established that site-specific factors, such as concentration of nitrates and seawater temperature, exerted a significant effect in shaping the spatial patterns of those *C. edule* pathogen distributions. Similar habitat conditions at different sample sites resulted in similar spatial patterns for *M. mercernariae*-like as well as for *Vibrio* spp. (*V. splendidus*) and 2-pathogen co-occurrence (including co-occurrence of both *Minchinia* species or either one *Minchinia* species and *Vibrio* spp.) in *C. edule* populations. This was further supported by the overrepresentation of coinfection with both *Minchinia* species or with *Vibrio* and either *Minchinia* species found in this study, which was non-consistent with the values expected by random chance. Although the three-pathogen co-occurrence with both *Minchinia* species and also *Vibrio* found in our study was consistent with the low values expected by random chance.

At a regional scale, no significant differences in pathogen distributions were detected between countries, suggesting that the differences found between both regions were consistent with that expected by random chance. Sequencing confirmed that the *Vibrio* spp. found in *C. edule* at both Irish and Welsh sites was best matched to *V. splendidus*. In accordance with our results, Costello et al. (unpublished data) also detected environmental DNA of *V. splendidus* in seawater samples along the Irish and Celtic Seas in 2018. Coscia et al. (2013) described how the *C. edule* larvae spawned in south Wales are capable of travelling west towards Ireland due to the Celtic Sea front residual current, thus, indicating the potential connectivity between certain *C. edule* pathogen groups in Wales and Ireland. Therefore, it is important to develop a better knowledge of the pathogen distribution not only at a regional scale but also at a local scale and improve our understanding of how site-specific factors can influence their spatial patterns.

At a local scale, the significant spatial variability in pathogen distributions between the sites was statistically associated with site-specific factors. The concentration of nitrate in the seawater was highlighted by the 1-factor models as the

best explanatory factor in driving the distribution of *Vibrio* spp. (*V. splendidus*), as well as the 2-pathogen co-occurrence in *C. edule*. Tralee - Castlemaine Harbour, a rural area with intensive agriculture and farming activity, showed the highest concentration of nitrate in seawater, which corresponded with the highest prevalence of 2-pathogen co-occurrence among the Irish and Welsh *C. edule* and was also associated with a high prevalence of *Vibrio* spp. (*V. splendidus*). In general, seawater concentration of nitrate at the Welsh sites was lower than at the Irish sites, except for Dyfi Estuary, which has a rich industrial heritage and has had reported problems in relation to water quality (www.naturalresources.wales). Correspondingly, Dyfi Estuary also exhibited a high prevalence of 2-pathogen co-occurrence and single detection of *Vibrio* spp. (*V. splendidus*). Anthropogenic inputs of nutrients may trigger changes in host abundance and distribution, an increase in host susceptibility, or shifts in pathogen populations, which may, eventually, cause mortalities of benthic fauna, including bivalves (Elliott and de Jonge, 2002; McKenzie and Townsend 2007; Johnson and Carpenter 2008; Johnson et al., 2010; Burdon et al., 2014). Increased nutrient loadings in the water may lead to several signs and symptoms of eutrophication, one of which is oxygen depletion that can stress the benthic fauna, making them more vulnerable to diseases (Elliott and de Jonge, 2002; Burdon et al., 2014). Bivalve molluscs growing in such waters can concentrate pollutants, such as are found in continuous and intermittent discharges, during the process of filter-feeding (Strubbia et al., 2016), and eutrophication is often associated with toxicants, which increase the individual's susceptibility to disease by impairing its defences (Lafferty et al., 2004). Several studies have suggested that organic loading and oxygen depletion may be a likely cause of mass cockle mortalities in the past (Desprez et al.,1992; Rybarczyk et al.,1996; Mudge and Bebianno, 1997). The extra organic input experienced by *C. edule* at those sites may impact host immune function while favouring pathogen proliferation and survival (Marcogliese, 2001). Previous studies have correlated nutrient enrichment with increases in the prevalence, severity, or distribution of infectious diseases in aquatic invertebrates and vertebrates (Lafferty, 1997; Coyner et al. 2003; Lafferty and Holt, 2003; Voss and Richardson 2006; Johnson et al. 2007; McKenzie and Townsend, 2007; Johnson and Carpenter, 2008). Higher concentrations of nutrients in seawater (primarily nitrogen and phosphorus) may lead to high biomass of seaweeds and algae, and the derived alginates are known to be consumed by *Vibrio* species (Lynch et al., 2021; Notaro et al., 2021).

Dale - Gann Flats showed the highest prevalence of *Vibrio* spp. (*V. splendidus*), with no other pathogen being detected, even though this site had a very low concentration of nitrate in the seawater. This site is next to Milford Haven Waterway, with the highest shipping activity registered in Wales (Welsh National Marine Plan, Welsh Government, 2020). The coastal development and shipping activity are often associated with toxic pollution, and toxicants can impair host defences (Lafferty et al., 2004). In addition to the shipping activity, the lowest concentration of dissolved oxygen registered in Dale - Gann Flats may have led to the high *Vibrio* prevalence. Low dissolved oxygen levels, harmful algal blooms, toxins and pollutants have been shown to promote infection by the bacterium *V. splendidus* (Soon and Zheng, 2020). Hypoxic conditions can also impact bivalve immune systems and interact with diseases, for example rendering bivalve hosts less efficient at eliminating bacterial cells (Macey et al., 2008). *Vibrio*, similar to other pathogen species, can behave opportunistically and exploit the vulnerability of the host under stressful conditions (Egerton et al., 2018).

Based on the 1-factor models, elevated seawater temperature was the best explanatory factor in driving the distribution of *M. mercenariae*-like in *C. edule*. A higher prevalence of *M. mercenariae*-like was detected in June 2018 (at Welsh sites) and March and April 2019 (at Irish sites). Nevertheless, *M. mercenariae*-like was not present as single detection either co-occurring with another pathogen in five out of the 13 sample sites. De Montaudouin et al. (2021) recently reviewed the parasitology of *C. edule* in the Atlantic Area and described that the recorded prevalence of *M. mercenariae* in *C. edule* has always been low. Ramilo et al. (2018) reported a prevalence of <10% with *M. mercenariae*-like parasite in Galician cockles, similar to as observed by Albuixech-Martí et al. (2021) in Irish *C. edule*. However, previous findings have suggested that a thermal tolerance range exists for haplosporidian species, which can influence their prevalence (Burreson and Ford, 2004; Engelsma et al. 2010; Lynch et al., 2014). Upper temperatures in warmer months have been associated with a higher prevalence of *Minchinia* species in *C. edule* (Carballal et al., 2020) and *Haplosporidium nelsoni* in eastern oyster *Crassostrea virginica* (Burreson and Ford, 2004). Warming could also affect parasites by influencing the distribution and life-history of hosts and potentially increase host susceptibility to infection due to thermal stress (Harvell et al., 2002; Callaway et al., 2012; Zgouridou et al., 2021).

None of the 1-factor models was conclusive in determining the distribution of *M. tapetis*. A potential explanation for the detection of low or null prevalence in *C. edule* at the Irish and Welsh sites is the possibility that mortalities of individuals infected with *M. tapetis* occurred. However, heavy infection intensity with *M. tapetis* in *C. edule* has never been recorded and no mortality outbreaks have been reported associated with this agent in the Atlantic Area (de Montaudouin et al., 2021). Even if *Minchinia* infection has not been directly associated with cockle mortalities, pathological responses in infected cockles with *Minchinia* are still considered to be detrimental to host survival (Elliott et al., 2012). Carballal et al. (2020) suggested that ocean warming might contribute to increasing the prevalence of *M. tapetis* in *C. edule* and, consequently, this agent may become a threat to cockle production.

Cork Harbour, Wexford Harbour and Dublin Bay, apart from Teifi Estuary, are the only sites that presented single detection of *M. tapetis* in *C. edule*. Moreover, Cork Harbour, Wexford Harbour and to a lesser extent Dublin Bay and Gower Bay (Burry), all known for their intense shipping activity, exhibited high detection levels of *M. mercenariae*-like. *C. edule* mass mortalities at Burry Inlet were first reported in the 1950s and 1960s (Hancock and Franklin, 1972) and since 2002 have recurred every year (Murray and Tarrant, 2015). Elliott et al. (2012) concluded that mortality events were the result of a combination of factors, including pathogens such as *Minchinia* spp. and host density dependence, and various extrinsic factors such as temperature and organic loading. Anthropogenic impacts, such as the pressure from increasing waterway degradation and pollution, have been associated with increased susceptibility of other bivalves such as *C. gigas* to pathogens through reduced immune functions (Moreau et al., 2015).

Dredging, including suction dredging activity, can disturb the sediment and affect bivalve host populations and other filter-feeding organisms and subsequently unbalance the interplay between infectious disease agents and their hosts in marine ecosystems (Cranfield et al., 1999; Cranfield et al., 2005; Rowley et al., 2014; Coen and Bishop, 2015). It has been previously reported in Ireland that exploited *C. edule* populations with high cockle densities were more exposed to infection and coinfection with *Minchinia* and *Vibrio* species (Albuixech-Martí et al., 2021). The high levels of microphotobenthic biofilms associated with cockle beds may also increase the persistence of infectious agents in the sediment (Carss et al., 2020). Accordingly, in

the Castlemaine Harbour (Tralee) and Gower Bay (Burry) fisheries, the prevalence of pathogens present was high compared with the other non-exploited sites. *C. edule* at Westport Bay, with the stock not commercially fished in recent years, showed low pathogen prevalence.

This study describes newly documented geographic ranges of *Minchinia* and *Vibrio* species in exploited and non-exploited *C. edule* populations, relating their spatial patterns to environmental and biological factors. The findings suggest that exposure of *C. edule* to a high concentration of nitrates, high temperatures and low levels of dissolved oxygen increased pathogen prevalence and may have affected host susceptibility. Likewise, shipping and cockle dredging activity at certain sites may have also influenced the host-pathogen systems. Overall, a complex pattern of interactions is expected between the host-pathogen systems and several habitat conditions (e.g., temperature, nutrients, anthropogenic impacts), which will influence the spatial distribution of host-pathogen systems. These findings highlight the need to examine pathogen distribution at different scales and emphasise the importance of site-specific monitoring to assess a number of potential drivers/inhibitors, given the highly dynamic nature of the estuarine environment. Findings from this study provide predicted scenarios that can facilitate pathogen emergence and possible outbreaks under certain environmental conditions, which are forecasted in the future. Investigating these relationships and modelling environmental drivers or inhibitors of host and pathogen distribution is essential for the sustainable management of coastal ecosystems, particularly in a changing marine environment with the increasing emergence of novel and known pathogens.

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Supplementary Material

Supplementary material Table S1: Overview of the 1-factor models explaining *Minchinia tapetis* prevalence (modelled as pathogen presence/absence) in *Cerastoderma edule.*

Model	Variable	Estimate (β) ± SE	AIC	ΔAIC	Akaike's weights (ω)
Null	Intercept	-3.2817 ± 0.8075	176.0	1.2	0.208
1-Factor	**Intercept**	**-3.4597 ± 0.9664**	**174.8**	**0.0**	**0.371**
	Temperature (°C)	**-0.8953 ± 0.6318**			
1-Factor	Intercept	-3.2884 ± 0.8030	176.4	1.6	0.166
	Dissolved O_2 (mmol/m^3)	0.5163 ± 0.4388			
1-Factor	Intercept	-3.2651 ± 0.7885	177.5	2.7	0.097
	Nitrates (mmol/m^3)	0.2396 ± 0.3546			
1-Factor	Intercept	-3.3127 ± 0.8320	177.9	3.1	0.081
	Salinity (PSU)	0.0831 ± 0.2611			
1-Factor	Intercept	-3.3437 ± 0.9470	178.0	3.1	0.078
	pH	-0.1052 ± 0.7029			

The 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) ($p(\chi^2) < 0.05$).

Supplementary material Table S2: Overview of the 1-factor models explaining *Minchinia mercenariae*-like prevalence (modelled as pathogen presence/absence) in *Cerastoderma edule.*

Model	Variable	Estimate (β) ± SE	AIC	ΔAIC	Akaike's weights (ω)
Null	Intercept	-1.640 ± 0.667	341.7	11.9	0.0023
1-Factor	**Intercept**	**-2.342 ± 1.693**	**329.8***	**0.0**	**0.8836**
	Temperature (°C)	**2.809 ± 1.032**			
1-Factor	Intercept	-2.056 ± 1.343	333.9*	4.2	0.1107
	Dissolved O_2 (mmol/m^3)	-1.368 ± 0.441			
1-Factor	Intercept	-1.7189 ± 0.6254	342.3	12.5	0.0017
	pH	0.5088 ± 0.4189			
1-Factor	Intercept	-1.61592 ± 0.65512	343.5	13.7	<0.001
	Salinity (PSU)	-0.07635 ± 0.17029			
1-Factor	Intercept	-1.66115 ± 0.69288	343.6	13.8	<0.001
	Nitrates (mmol/m^3)	-0.09559 ± 0.24437			

The 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) ($p(\chi^2) < 0.05$).

Supplementary material Table S3: Overview of the 1-factor models explaining *Vibrio* spp., most likely *V. splendidus*, prevalence (modelled as pathogen presence/absence) in *Cerastoderma edule.*

Model	Variable	Estimate (β) ± SE	AIC	ΔAIC	Akaike's weights (ω)
Null	Intercept	-0.2406 ± 0.4421	394.7	29.2	<0.001
1-Factor	**Intercept**	**-0.1331 ± 0.5181**	**365.5***	**0.0**	**1**
	Nitrates (mmol/m^3)	**1.4580 ± 0.3256**			
1-Factor	Intercept	-0.08517 ± 0.52312	382.8*	17.2	<0.001
	Salinity (PSU)	-0.63442 ± 0.18206			
1-Factor	Intercept	-0.07271 ± 0.51438	394.0	28.5	<0.001
	pH	-0.60553 ± 0.39499			
1-Factor	Intercept	-0.2076 ± 0.4619	396.1	30.6	<0.001
	Temperature (°C)	0.2643 ± 0.3328			
1-Factor	Intercept	-0.2375 ± 0.4725	396.2	30.7	<0.001
	Dissolved O_2 (mmol/m^3)	0.2033 ± 0.2996			

The 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) ($p(\chi^2) < 0.05$).

Supplementary material Table S4: Overview of the 1-factor models explaining 2-pathogen co-occurrence (modelled as pathogen co-occurrence presence/absence) in *Cerastoderma edule.*

Model	Variable	Estimate (β) ± SE	AIC	ΔAIC	Akaike's weights (ω)
Null	Intercept	-2.1336 ± 0.5938	260.9	21.2	<0.001
1-Factor	**Intercept**	**-2.2468 ± 0.5646**	**239.7***	**0.0**	**0.977**
	Nitrates (mmol/m^3)	**1.5342 ± 0.4250**			
1-Factor	Intercept	-2.1018 ± 0.5848	247.2*	7.5	0.023
	Salinity (PSU)	-0.9909 ± 0.3017			
1-Factor	Intercept	-2.1701 ± 0.4724	257.6*	17.9	<0.001
	Dissolved O_2 (mmol/m^3)	0.7580 ± 0.3225			
1-Factor	Intercept	-2.1364 ± 0.7707	261.6	22.0	<0.001
	pH	-0.6971 ± 0.6305			
1-Factor	Intercept	-2.1247 ± 0.5542	262.8	23.1	<0.001
	Temperature (°C)	-0.1953 ± 0.4996			

The 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) ($p(\chi^2) < 0.05$).

CHAPTER 7

Conclusions and Implications

Key conclusions – overview:

- The host-parasite interplay in *Cerastoderma edule* should be viewed as a pathosystem defined by the reciprocal interaction between the host, the internal pathogen/parasite community, and the external environment.

- All the screened cockles in this study were identified as *C. edule*, with no presence of lagoon cockle *Cerastoderma glaucum* or hybrids.

- Species-specific PCRs were developed for the specific detection of two significant haplosporidian species, *Minchinia tapetis* and *Minchinia mercenariae*-like.

- A novel geographic range of *Minchinia tapetis* and *M. mercenariae*-like in *C. edule* was detected along the Irish coastline in the Irish and Celtic Seas.

- Biological and environmental factors played a key role in driving *Minchinia* infections in *C. edule* spatially and temporally.

- Co-occurrence of microbial assemblages within *C. edule*. Simultaneous infections with haplosporidians and *Vibrio* spp. were described for the first time in *C. edule* populations along the Irish coast. No microsporidian species nor OsHV-1 μVar were detected.

- Single infections with haplosporidian or *Vibrio* species were more common in *C. edule* than coinfected individuals.

- Coinfections with Haplosporidia and *Vibrio* in *C. edule* resulted because the same risk factors, biotic and abiotic, shared by the two pathogens, increased the probability of infection by both pathogen groups.

- *Minchinia tapetis* and *M. mercenariae*-like in *C. edule* showed a positive two-way association between both species regardless of sample time and sample site.

- Identical strains of *Vibrio splendidus* were found in *C. edule* and, for the first time, in shorebird faecal samples, thus, highlighting that *C. edule* ingestion and mobility in seabirds play a role in the transmission and persistence of *Vibrio* species.

- Our findings further support the role of the sediment as an environmental reservoir for *Vibrio*, where the bacteria can find a favourable environment for overwintering.

- Haplosporidia transmission to shorebirds via *C. edule* ingestion was not detected, nor the presence of Haplosporidia in sediment, providing new insight into its capacity of persistence outside the host.

- *Minchinia tapetis* and *M. mercernariae*-like as well as *Vibrio* spp., most likely *V. splendidus,* and 2-pathogen co-occurrence in exploited and non-exploited *C. edule* populations were reported on a wider geographic range across Ireland and Wales.

- Environmental and anthropogenic stressors (high concentration of nitrates, warm temperatures, low levels of dissolved oxygen, shipping and cockle dredging activity) may have increased pathogen prevalence and affected host susceptibility, shaping the observed spatial patterns of pathogen occurrence in *C. edule* populations.

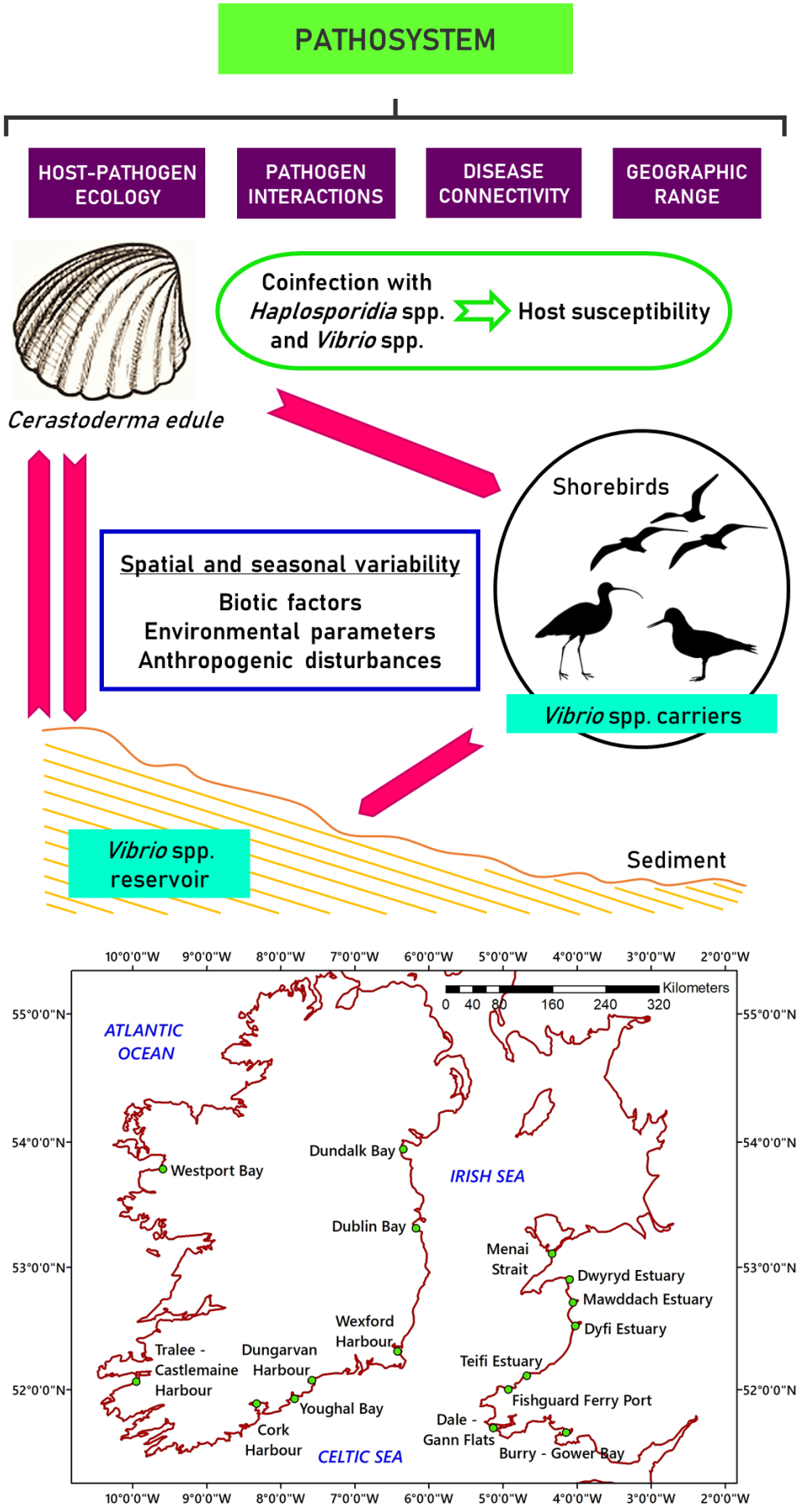

Figure 1: Key study findings.

7.1. *Cerastoderma edule* as a pathosystem representative of the complexity of host-pathogen interplay in the natural systems

The integration of the host-pathogen system within the environment as a pathosystem provides a better understanding of the intricate interactions occurring and affecting pathogen diversity, prevalence, transmission, and distribution, especially in a changing marine environment. However, connections between the community structure and the emergence, spread, and persistence of marine diseases are very rarely made.

As far as could be ascertained during this study, the concept of the pathosystem has never been associated with cockles. The wide range of infectious agents occurring in *C. edule*, their host-parasite interactions and their impact on non-host populations, as well as the environmental influence shape a complex ecosystem. Accordingly, the host-parasite interplay should be viewed as a pathosystem, i.e., an ecological subsystem within an environment, which is defined by the reciprocal interaction between the host, the parasite, and the environment (Sullivan et al., 2013). A better understanding of this holistic pathosystem approach may shed light on how parasites and pathogens affect directly and indirectly host individuals and population health and dynamics, and, in turn, how to influence the community structure. In that sense, *C. edule* can serve as an ideal model to highlight the activity of agents causing emerging/novel infectious diseases in fluctuating environments and investigate the role of environmental stress on host-pathogen interplay. *C. edule* is also an ecosystem engineer which provides regulating ecosystem services, such as bioturbation, nutrient recycling and carbon sequestration (Carss et al., 2020). The aforementioned regulating services can be imperative in coastal and restoration studies, showing the wider benefits of a better understanding of the *C. edule* pathosystem in order to keep a healthy ecosystem that persists, and maintains vigour, organization and resilience to change. The integrative approach taken in this thesis, in turn, might be extended to other bivalve species and the problems facing those populations, which would have important consequences for the sustainability of wild and cultured bivalve populations and the function of nearshore ecosystems.

7.2. Host-pathogen ecology using *Cerastoderma edule* as a model organism

Pathogens and micro-parasites in *C. edule* have had less attention compared to macroparasites, due to their challenging detection (Burreson and Ford, 2004; Hartikainen et al., 2014). Despite that, previous studies have highlighted the importance of Phylum Haplosporidia in bivalve health studies (Burreson and Ford, 2004; Lynch et al., 2013; Lynch et al., 2014; Arzul and Carnegie, 2015; Ramilo et al., 2018; Lynch et al., 2020).

In our study, a detailed examination of the microbial community with newly developed species-specific primers revealed a novel geographic range of two haplosporidian species, *Minchinia tapetis* and *Minchinia mercenariae*-like, in *C. edule* along the Irish coastline in the Irish and Celtic Seas. These findings, thus, further support the possibility that haplosporidian species have a greater geographic range than originally documented. The characterisation of both *Minchinia* species in *C. edule* is recent. *M. tapetis* was identified in *C. edule* for the first time in the Netherlands (Engelsma et al., 2011) and, subsequently, in Wales (Elliott et al., 2012) and Spain (Carballal et al., 2020), while *M. mercenariae*-like was recently detected in *C. edule* in Spain (Ramilo et al., 2018) and, subsequently, in Ireland (Lynch et al., 2020). Moreover, very little is known about the life cycle of *Minchinia* species. The observation of spore-like stages in the connective tissues of *C. edule* in this study adds evidence to the recent description of spores of *M. mercenariae*-like in Irish *C. edule* (Lynch et al., 2020), which have not been observed either in the *M. mercenariae*-like parasite (Ramilo et al. 2018) or in *M. tapetis* infecting *C. edule* in Galicia (NW Spain) (Carballal et al., 2020). The new findings of spore-like stages, presumably the transmission stage as seen in *Haplosporidium nelsoni* (Ford et al., 2018), in *C. edule* and the availability of molecular diagnostics should encourage future investigations into the *Minchinia* life cycle. A better understanding of the *Minchinia* life cycle will provide a better comprehension of the pathogen transmission and, consequently, a more accurate approach in the disease management in *C. edule*, but also in other hosts such as clams. Future research should address if the pathogen must complete its life cycle by producing spores, or if even in the absence of spore production, there continues to be a quantity of infective particles sufficient to cause infections in host individuals. In addition to the use of histopathology, further research on haplosporidian life cycles could be addressed using a combination of molecular and

experimental approaches. In the last years, there has been an increasing development of molecular technology which provides exciting possibilities for the study of microparasites, which historically have been difficult and costly to sample. These molecular screening techniques have enabled high-throughput rapid-detection of pathogens within molluscs (e.g., Romalde et al., 2014; Thompson et al., 2015). However, there still are many haplosporidian species whose DNA sequence has not been described yet, as supported by the finding of a potential non-identified haplosporidian species in our study. Therefore, further research in the development of more specific probes to identify novel/emergent haplosporidians will contribute to determining the species diversity and their geographic distribution. The development of specific PCRs will complement other available diagnostic methods such as generic PCRs, histology, ISH, and immunochemistry.

Minchinia detection in *C. edule* also adds evidence to the potential infection of these *Minchinia* species to other bivalve species (Engelsma et al., 2011; Elliott et al., 2012; Ramilo et al., 2018; Lynch et al., 2020; Carballal et al., 2020), apart from clams, their natural host (Navas et al. 1992; Villalba, et al. 1993; Azevedo, 2001; Ford et al., 2009), which can have implications for the biosecurity and management of the bivalve industry. The host-jumping from the natural host to another susceptible species, which may not have the defences to defeat the infection, might increase the pathogen dispersion and transmission, and, eventually, trigger host abundance declines and population crashes. Given the current changing environment, the emergence of infectious agents in hosts and/or areas that were not present before will probably continue over the foreseeable future. Therefore, the identification of those emergent/novel infectious agents should be a priority for providing an early warning of potential emergence and outbreaks of previously non-problematic infectious agents.

The knowledge of how the physical and biological processes can influence *Minchinia* distribution is scarce, especially under environmental stress (de Mountaudouin et al., 2021). Host condition was determined to have a key role in shaping host-pathogen interactions in this study, with smaller and older *C. edule* being more vulnerable to the infection. Likewise, environmental drivers (higher dissolved oxygen, lower temperature, and lower salinity in seawater) may have affected both

spatial and temporal patterns of haplosporidian infection in *C. edule* distribution. Therefore, the *Minchinia* emergence may become more frequent over time under the increasing environmental stress. Climate change, in fact, might be contributing to the emergence, translocation and virulence of diseases, parasites and pathogens (Callaway et al., 2012; Soon and Zheng, 2020; Zgouridou et al., 2021). Rapid changes derived from climate change, such as warming in coastal regions and ocean acidification, can boost marine pathogens that will impact the immune system of bivalves and weaken their tolerance to extreme environmental conditions (Groner et al., 2016; Turner et al., 2016; Cao et al., 2018; Avdelas et al., 2021). As a result of physiological malfunction, mass mortalities and high losses in hatcheries as well as in natural beds and spat are expected on a national and global scale (Groner et al., 2016; Turner et al., 2016; Cao et al., 2018; Avdelas et al., 2021). Even though these *Minchinia* species have never been associated with abnormal cockle mortality (de Mountaudouin et al., 2021), *M. tapetis* infection has been associated with heavy haemocytic infiltration around parasite foci in *C. edule* (Carballal et al., 2020). This heavy inflammatory reaction could cause host damage and weakness, favouring other pathological conditions (Carballal et al., 2020).

Additionally, diseases that are assumed to peak in a single season may follow atypical infection patterns due to climatic change (Coen and Bishop, 2015) and, thus, one-time sampling may miss critical periods. Enhanced longitudinal studies assessing the occurrence of infectious diseases in various host populations, along with more specific approaches that focus on the actual causative agents of these existing and novel diseases, must be developed to detect both the frequency and range of disease epizootics, especially on species that are bioindicators and ecosystem engineers, such as *C. edule*. Future research, thus, should focus on long-term studies that consider host-pathogen dynamics and interactions within the environment, as a pathosystem, and assess the impact of the environmental stress on the functioning of wild and fished bivalve populations.

7.3. Pathogen community in *Cerastoderma edule*: diversity, interactions and impacts in cockle health

This study gives an overview of the microbial community in *C. edule*, providing evidence of the interactions occurring within the hosts. Simultaneous infections with haplosporidians and *Vibrio* spp. were detected in wild and fished *C. edule* populations, and a positive two-way association between *M. tapetis* and *M. mercenariae*-like was statistically confirmed. Given the increasing emergence of infectious agents due to climate change and anthropogenic disturbances (Coen and Bishop, 2015), it is particularly important to consider the potential interactions between the different agents within the host and the effects that may have on the bivalve populations. Even though it is well-known that *C. edule* can be the host of multiple pathogens and micro-parasites (Longshaw and Malham, 2013), the causes of coinfection and the interactions between those infectious agents have been neglected. However, a better understanding of the mechanisms underlying the interactions between the infectious agents and the synergistic or antagonistic effects of simultaneous infections within the host would be beneficial to ensure that biologically relevant decisions are made in managing any wild and harvested cockle populations. Future research should address how simultaneous infections can affect bivalve immunity and their susceptibility to further infectious agents. Even though a pathogen might not be problematic on its own, the immune suppression triggered by a primary infection could facilitate the infection by another pathogen group. Therefore, the detrimental effect of simultaneous infections on bivalve health needs to be addressed and considered in the management of bivalve beds and stocks. Given the rising bivalve farming worldwide, further studies on the microbiological quality of the product and bivalve immunity will expand our understanding of their defence mechanisms, which will improve their management from both a human health perspective and for reducing economic losses. Similarities in the host environment or susceptibility can also cause correlations in the risk of infection between two infectious agents, even though involved agents do not interact biologically (Hellard et al., 2015; Vaumourin et al., 2015). As it was observed in this study with the double infected individuals with Haplosporidia and *Vibrio*. Consequently, future research should also focus on how the host condition and habitat could facilitate simultaneous infections, and how parasite burdens that are considered

harmless in favourable environmental conditions could become stressful harmful agents in unfavourable environmental conditions.

Accordingly, future research should move from the study of one host-one pathogen systems towards more complex systems considering the pathogen community within the host and their relationship with the environment. Further research to reveal the underlying mechanisms of the pathogen interactions within the host and the role that host exposure and habitat play in it, is crucial in order to develop a better understanding of the relationship between pathogen diversity, transmission and disease.

7.4. Disease connectivity of pathogens associated with *Cerastoderma edule* in mudflats

The previous pathological studies of marine bivalve molluscs have mainly enhanced our understanding of how disease shapes marine molluscan ecology at the population level but have seldom considered the impacts of disease at higher levels of ecological organization. Nevertheless, hosts exist within diverse ecological communities, and ecological interactions undoubtedly influence the transmission and impact of aetiological agents (Dobson, 2004; Hall et al., 2007). Even though it is well-known that infectious agents can be trophically transmitted via predator-prey interactions (Coen and Bishop, 2015), the trophic transmission of microbial infections in aquatic birds and their influence on disease dynamics and dissemination are difficult to establish.

The findings of this study provided the first evidence of transmission of *Vibrio splendidus* to shorebirds through consumption of infected *C. edule*. *V. splendidus* detection in bird faeces further support the carrier role that migratory birds play in the dissemination of *Vibrio* species, reported previously for other *Vibrio* species important for human health, such as *V. cholerae* (Halpern et al., 2008; Rodríguez et al., 2010; Fernandez-Delgado et al., 2016; Laviad-Shitrit et al., 2019), *V. parahaemolyticus*, *V. vulnificus* and *V. mimicus* (Buck, 1990; Miyasaka et al., 2006; Fu et al., 2019). Future research should examine the infection success this pathogen can have on shorebird populations, or if on the contrary, *Vibrio* just pass through the digestive tract, being

viable when excreted, which will determine the range of pathogen dispersal and transmissibility. The use of both *in vitro* and *in vivo* models should be considered in future studies to determine the impact of this pathogen on shorebirds at an individual and a population level, as well as to identify those biological and environmental factors that influence the expression of disease.

Environmental conditions are often directly linked to disease emergence, persistence, and spread, and must be addressed to successfully prevent and control disease (Friend et al., 2001; Harvell et al., 2002; Newman et al., 2007). Climate warming may affect host-pathogen interactions by increasing pathogen development rates, transmission, and the number of generations per year; relaxing overwintering restrictions on pathogen life cycles; and modifying host susceptibility to infection (Harvell et al., 2002). Accordingly, in our study, higher *Vibrio* prevalence in bird faeces was observed preferentially during the warmer months. The high seawater temperature is a key driver of the proliferation of *Vibrio* and, thus, ocean warming can promote the proliferation and spread of vibrios and contribute to the ongoing range expansions (Vezzulli et al., 2012; Vezzulli et al., 2013; Vezzulli et al., 2016). Consequently, *Vibrio* species which currently are non-problematic for shorebird populations can start more frequently being transmitted to shorebirds and become challenging for the survival of susceptible individuals. In addition, the effect of the environmental stress on the bird condition and their immune system can facilitate the infection of vibrios, promoting long-distance pathogen dissemination. The increasing dispersion of *Vibrio* by shorebirds might have severe consequences for areas where it may be introduced. These introduced pathogens may spread to naïve host populations or other naïve host species, and, eventually, they can become a threat to humans if vibrios continue their trend of proliferation and spread to coastal areas, that people depend on for food, recreation, and their well-being. Therefore, monitoring programs for the prevalence and severity of wildlife diseases and their impacts must be implemented for a wider range of natural systems in order to early detect and predict changes and control outbreaks and population crashes. This will have implications for aquatic bird health, but also for human health, as the aquatic birds may act as sentinels for contamination of the marine ecosystems.

The higher occurrence of *Vibrio* spp. in sediment samples further supports the role of the sediment as an environmental reservoir of *Vibrio*, from which the bacteria

can emerge when environmental conditions became favourable (Vezzulli et al., 2009; Vezzulli et al., 2015; Azandégbé et al., 2010). The increasing coastal development and some of the fishing practices such as dredging or trawling can intensify the sediment disturbance and, consequently, increase the pathogen transfer to the water column and the probability of host contact with the infectious agents. Therefore, the presence of vibrios in the physical environment (sediment and water column) should also be monitored, as climate change and increasing anthropogenic disturbances may promote new threats. Overall, the presence of pathogens in the sediment may have an impact not only on *C. edule* but also on the entire infaunal community within the sediment, which influence ecosystem services such as bioturbation, nutrient recycling or carbon sequestration (Carss et al., 2020).

Many of the examples of how infectious agents modify the community structure and even the whole ecosystems and their services have come from the study of macroparasites. However, our knowledge remains largely scant for many molluscan host-parasite systems in intertidal environments, in particular those involving microparasites. The use of *C. edule* and its microbial community as a pathosystem model, thus, may shed new light on the transmission of diseases in bivalve populations and the critical role of passive carriers, reservoirs, and vector species in an ecological context. The next step should be the examination of the role of plankton in the dissemination of selected cockle pathogens, as this constitutes a major component of cockle diets (Longshaw and Malham, 2013), in order to provide a complete picture of the infection sources and the potential connectivity between them in the pathosystem model *C. edule*.

Therefore, wildlife disease surveillance efforts from multiple ecological levels of organization will need to be combined and analysed to obtain a better understanding of the pathogen transmission through the trophic web and the impacts on the hosts and ecosystems. Environmental conditions should be considered in wildlife monitoring in order to develop better management and conservation strategies and minimise the risk of pathogen dissemination, as well as provide a robust early warning system for larger-scale marine perturbations. This knowledge will provide a more accurate overview of the ecosystem health, which is crucial for an appropriate approach to conservation and resource management.

7.5. Spatial distribution of pathogens associated with *Cerastoderma edule* along the Irish and Welsh coasts

Coastal marine environments are extremely vulnerable to change (Holt et al., 2010) and the predicted alteration of coastal waters (warming, reduced salinity, etc.) can impact the distribution, life history and physiological status of pathogens, hosts, and vectors (Gallana et al., 2013; Rowley et al., 2014). Indeed, a faster emergence of pathogen outbreaks, especially the thermopile species, is expected under climate change (Zgouridou et al., 2021). Moreover, most bivalve species, growing mainly in estuaries and coastal waters, are often exposed to polluted urban or agricultural wastes, which may accelerate the increase of pathogen contamination, causing infections in the majority of bivalve species (Moullec et al., 2019). Accordingly, this study describes newly documented geographic ranges of *Minchinia* and *Vibrio* species in exploited and non-exploited *C. edule* populations across Ireland and Wales, relating their spatial patterns to high concentrations of nitrates, warm temperatures, and low levels of dissolved oxygen, as well as to shipping and cockle dredging activity. Our findings revealed the site-specific variability in the pathogen distributions and emphasised the importance of site-specific monitoring, given the highly dynamic nature of the intertidal environments. Better knowledge of the spatial patterns of those pathogens at different scales may provide an early warning of the emergence and outbreaks of previously non-problematic infectious agents which can now become a threat under the current anthropogenic pressure.

Studies such as ours provide predicted scenarios that can facilitate pathogen emergences and possible outbreaks under environmental stress and anthropogenic disturbances. Given most coastal waters are typically affected by suites of anthropogenic pollutants and inputs, it is often difficult to identify only one specific cause of deteriorating health or disease outbreak (Harvell et al. 1999; Elliott et al., 2012; Zgouridou et al., 2021). Future work should include assessing how habitat degradation and pollutant inputs, often brought about by human activity, can facilitate disease emergence and outbreaks, particularly among sentinel and bioindicator species such as *C. edule*. The early detection of pathogen emergence in a bioindicator species may alert of potential anthropogenic impacts promoting the infection and may avoid further damage to the community.

In the current context of climate change, increased ocean pollution and intensifying aquaculture activities, the surveillance and mitigation of disease emergence should be a priority. Improving surveillance requires fast, accurate diagnosis, forecasting disease risk assessment and real-time monitoring of environmental conditions promoting disease. Further development of molecular techniques such as metagenomics, barcoding or next-generation sequencing will improve the diagnosis and detection of microbial pathogens with a poorly understood spatial distribution. Moreover, a diverse array of new remote sensing technologies has been developed, including optical instrumentation on satellites, aircraft, moored and floating systems, gliders and autonomous vehicles, that may measure environmental variables as well as aspects of habitat structure and, in some cases, real-time data streams. Those new technologies when accompanied by monitoring of disease, provide powerful opportunities to assess relationships between environmental change and disease outbreaks, at large spatial and temporal scales. The overarching aim should be to provide region-wide adaptation strategies for the benefit of coastal communities. Protecting coastal communities will not only be beneficial for the species inhabiting there but also to the entire marine ecosystem since coastal communities are responsible for key ecosystem services.

7.6. Future research questions

The pathological studies of cockles have been often overlooked compared with other cultured bivalve species. Nevertheless, a species such as *C. edule*, growing and being harvested in the wild, is particularly important in order to study how the current fluctuating environmental conditions and the increasing anthropogenic stressors affect the disease dynamics in the nearshore areas. Therefore, *C. edule*, as seen in this study, may be a key bioindicator species for the early detection of emergent infectious agents. Likewise, the concepts gained from the *C. edule* pathosystem might be applied to other bivalve species and the changing environmental contexts forecasted in the future.

In short, through this study, we highlight that the use of *C. edule* as a model pathosystem provides crucial information on the host-pathogen interplay and its

interaction with the environment. This study has raised new important questions regarding the detrimental effect of emergent infectious agents in *C. edule* populations. Future research, thus, should investigate further how emergent pathogens and their interactions can affect *C. edule* health and trigger mortalities under environmental stress. However, the studies with molluscs are often based on spatial and temporally limited sampling and wide-ranging environmental conditions, thus, a horizon scanning approach with longitudinal monitoring of emergent pathogens in wider geographical areas should be considered in the future. Likewise, the sampling design and the need for replication at finer scales are fundamental for parasitological studies, given the high natural variation in the prevalence of infection at a finer scale. Natural variation in the presence and prevalence of multiple pathogens can be expected at the microscale sampling frame within the bivalve beds. Therefore, a clustering sampling design that considers several random clusters within the same sample site would give a more accurate view of the natural variability of the multiple pathogen groups present in a specific site. Replicated multi-cluster on each sample site will give information in terms of intra-cluster correlation coefficient and the between-cluster variance. Along with descriptive studies generating hypotheses about the biotic and environmental controlling factors that may lead to disease outbreaks and mass-mortality events, experimental pathology studies should be conducted to establish the causation of mortality. Overall, the limited number of experiments examining cause-effect relationships between disease and ecological change at larger spatial scales and different levels of ecological organization remains a major research gap.

This thesis provides insight into the role of infectious agents as essential components of the ecosystems, but there are still many gaps in the knowledge of the underlying mechanisms driving the host-pathogen systems. Hence, a better understanding of the infectious agents as an integral part of the pathosystems and their influence on the assembly, functioning and stability of communities and ecosystems is required.

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Appendix

Peer-reviewed publications

Albuixech-Martí, S., Lynch, S. A., Culloty, S. C., 2020. Biotic and abiotic factors influencing haplosporidian species distribution in the cockle *Cerastoderma edule* in Ireland. *Journal of Invertebrate Pathology* 174, 107425. 10.1016/j.jip.2020.107425.

Albuixech-Martí S., Culloty S.C., Lynch S.A., 2021. Co-occurrence of pathogen assemblages in a keystone species the common cockle *Cerastoderma edule* on the Irish coast. *Parasitology* 1–15. 10.1017/S0031182021001396.

Albuixech-Martí, S., Lynch, S. A., Culloty, S. C., 2021. Connectivity dynamics in Irish mudflats between microorganisms including *Vibrio* spp., common cockles *Cerastoderma edule*, and shorebirds. *Scientific Reports* 11, 22159. 10.1038/s41598-021-01610-x.

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

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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.

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

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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., 2019), *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., 2019).

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., 2019) 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 (Fig. 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).

Cockles of all available sizes were collected by hand raking from the intertidal area of each site at spring tide (0.5–1 m) 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

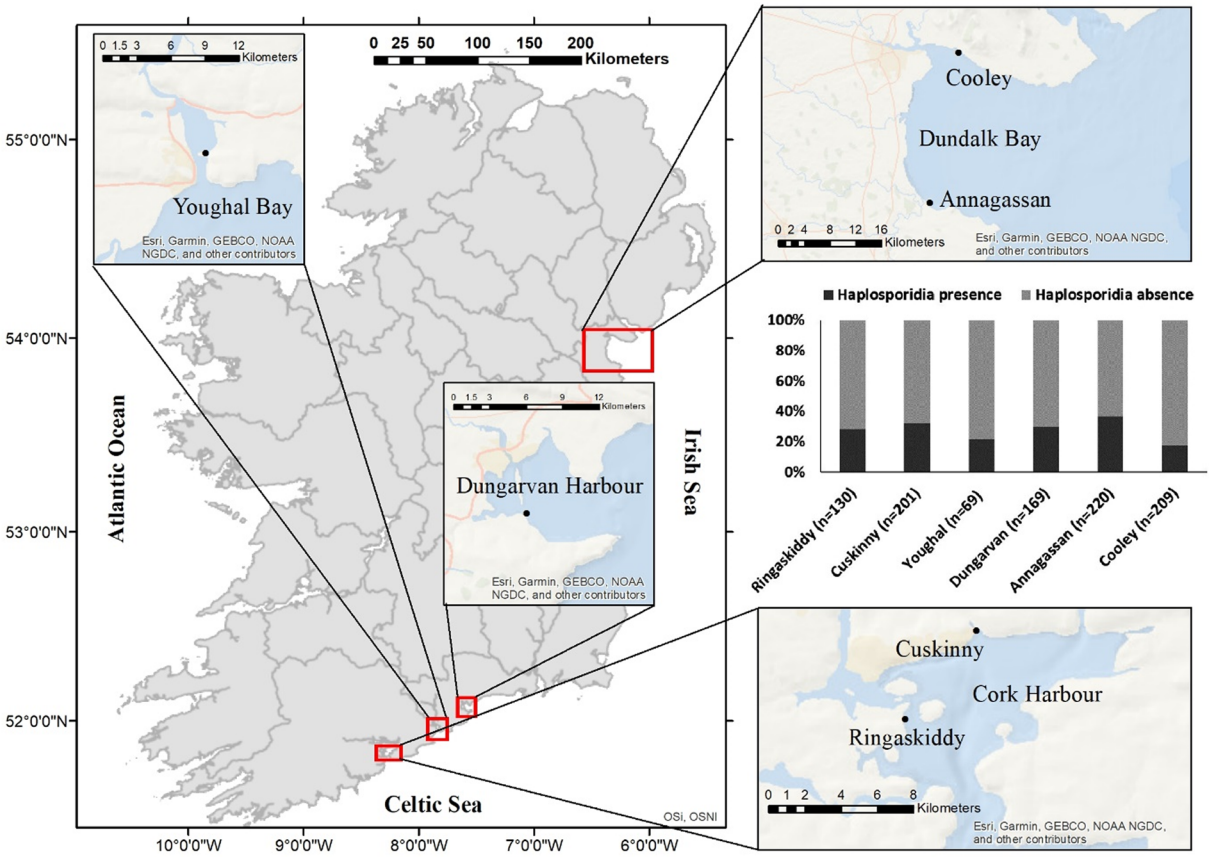

Fig. 1. Map of Ireland highlighting the cockle sample sites and the mean haplosporidian presence and absence (%) at each sample site (Ringaskiddy and Cuskinny in Cork Harbour; Youghal Bay; Dungarvan Harbour; Annagassan, and Cooley in Dundalk Bay).

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.

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 5 µm 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.

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

Sites		Coordinates	Trophic status	Description
Covrk 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	51°51′29.94″N 8°15′49.50″W		Cuskinny is a Marsh Nature Reserve supporting an array of habitats within a relatively small area, for a diverse community of wildlife.
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).
	Cooley	54° 0′16.84″N 6°17′43.17″W	Intermediate polluted	Annagassan and Cooley are oppositely located, with Cooley mainly affected by freshwater runoff from Castletown River and Flurry River due to its proximity.

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.

2.2.3. 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 20 µl of the reaction mixture containing 4 µl of 5x green buffer, 4 µl of dNTPs (1.25 mM), 0.2 µl of each primer (ITS-for / ITS Ce-R / ITS Cg-R, Table 3), 0.1 µl of GoTaq polymerases, 10.3 µl of ddH_2O and 1 µl of total genomic DNA under the following conditions: 95 °C for 3 min (1 cycle), 95 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s (35 cycles) and 72 °C for 5 min. Electrophoresis of the amplification products was conducted in a 2% agarose gel. The expected amplified product (amplicon) size for *C. edule* was 190 bp, and for *C. glaucum* 470 bp 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 20 µl of the reaction mixture containing 4 µl of 5x green buffer, 4 µl of dNTPs (1.25 mM), 0.4 µl of each primer (Table 3), 0.2 µl of GoTaq polymerases, 10 µl 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 min, 35 cycles at 95 °C for 30 s, 48 °C for 30 s, and 72 °C for 30 s with final extension at 72 °C for 5 min. 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 0.2 µl, 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

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

	Primer sequence (5′–3′)			
Primer pair	Forward	Reverse	Product size (bp)	Primer specificity
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

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.

presence of other haplosporidian species occurring in the samples not detected by the newly designed primers.

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 0.5 m 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 et al., 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 ΔAIC values ≤ 2 (i.e. not statistically different from the best performing model with ΔAIC = 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 300 bp, 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 318 bp, 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. (2019). Only in one sequence, the query coverage dropped to 75%, with a query length of 393 bp 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 170 bp, 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. (2019). 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 165 bp, 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 165 bp, 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.

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 (Fig. 2; Lynch et al., 2019), 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.

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

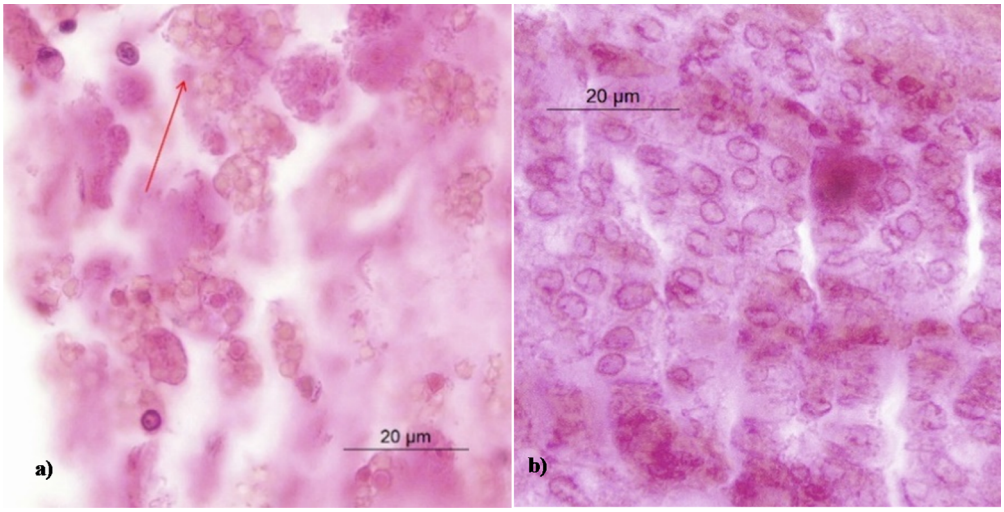

Fig. 2. a) Multiple haplosporidia-like sporonts in the connective tissues of *Cerastoderma edule*; and b) a large number of haplosporidian spores in the mantle tissue of *C. edule*.

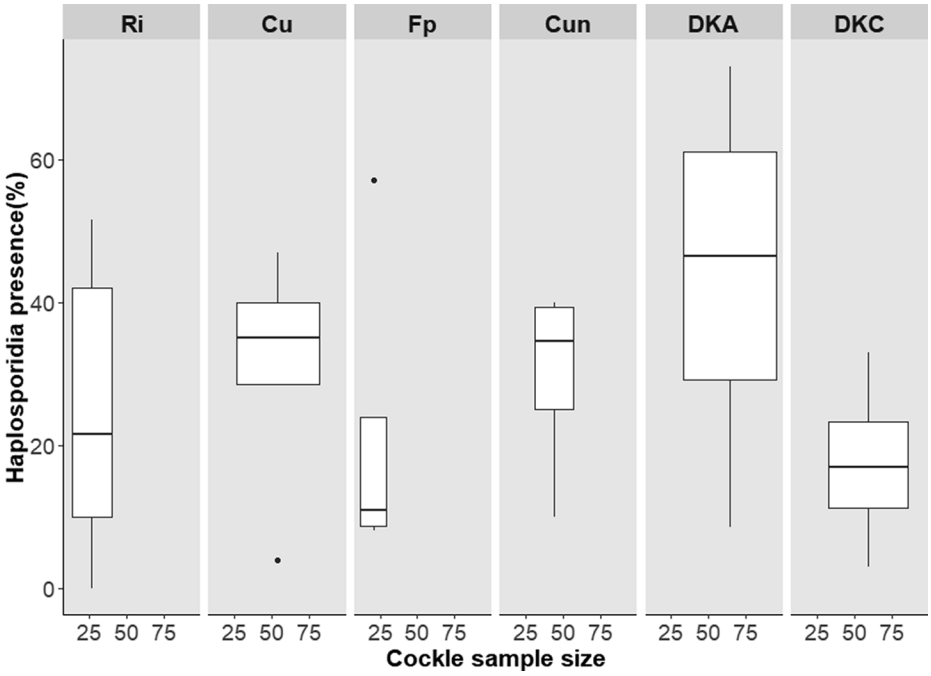

Fig. 3. Haplosporidian distribution in cockles collected at each sample sites (Ri: Ringaskiddy and Cu: Cuskinny, nested sites in Cork Harbour; Fp: Youghal Bay; Cun: Dungarvan Harbour; DKA: Annagassan and DKC: Cooley, nested sites in Dundalk Bay), combining the data from all the seasons. The boxplot compactly 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 (interquartile range, i.e. distance between the first and third quartiles) from the hinge.

(28.3 ± 21.0%), Youghal (21.7 ± 23.6%) and Cooley with the lowest mean occurrence (17.3 ± 17.1%) (Fig. 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.

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 (Fig. 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.

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).

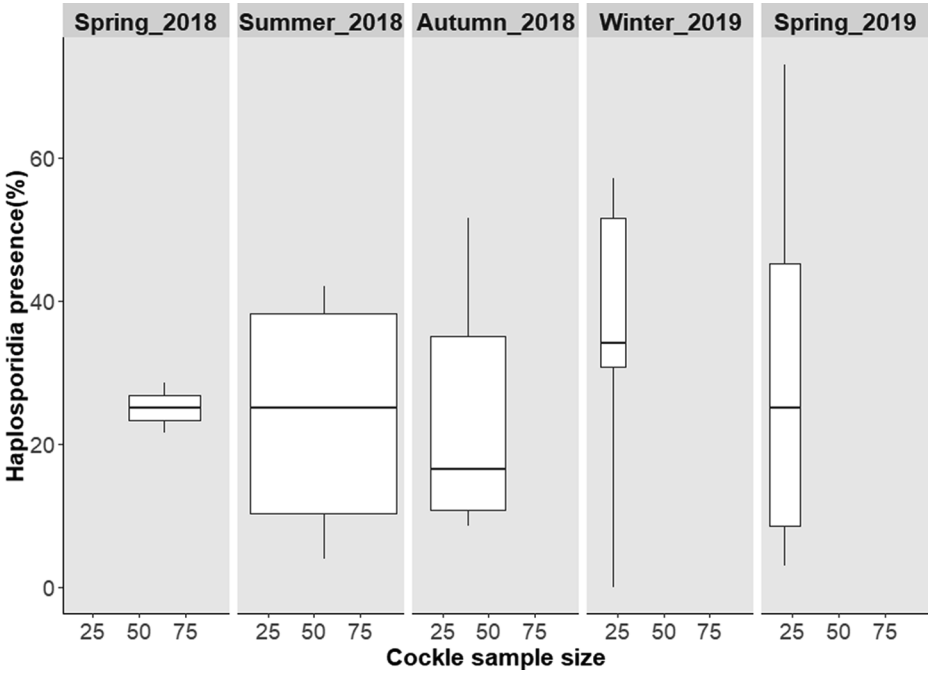

Fig. 4. Haplosporidian distribution in cockles collected from different seasons (spring 2018, summer 2018, autumn 2018, winter 2018/19, spring 2019), combining the data from all the sample sites. The boxplot compactly displays the distribution of the response variable - Haplosporidia presence (%) -. 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.

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 ± 3.3 mm) to winter 2018/19, when the lowest mean height (25.3 ± 3.6 mm) was recorded, and greater cockles were subsequently sampled in spring 2019 (28.4 ± 3.2 mm). 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).

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; ΔAIC ≥ 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.

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).

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

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

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).

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

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).

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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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2020.107425.

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Co-occurrence of pathogen assemblages in a keystone species the common cockle *Cerastoderma edule* on the Irish coast

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Research Article

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Abstract

Despite coinfections being recognized as the rule in animal populations, most studies focus on single pathogen systems. Pathogen interaction networks and the drivers of such associations are lacking in disease ecology studies. Common cockle *Cerastoderma edule* populations are exposed to a great diversity of pathogens, thus making them a good model system to investigate. This study examined the diversity and prevalence of pathogens from different taxonomic levels in wild and fished *C. edule* on the Irish coast. Potential interactions were tested focussing on abiotic (seawater temperature and salinity) and biotic (cockle size and age, and epiflora on shells) factors. No Microsporidia nor OsHV-1μVar were detected. Single infections with Haplosporidia (37.7%) or *Vibrio* (25.3%) were more common than two-pathogen coinfected individuals (9.5%), which may more easily succumb to infection. Fished *C. edule* populations with high cockle densities were more exposed to infections. Higher temperature and presence of epiflora on cockle shells promoted coinfection in warmer months. Low seawater salinity, host condition and proximity to other infected host species influenced coinfection distribution. A positive association between two *Minchinia* spp. was observed, most likely due to their different pathogenic effect. Findings highlight the major influence that ecological factors have on pathogen interactions and host–pathogen interplay.

Introduction

Animal hosts are exposed to complex pathogen assemblages that ultimately form a dynamic community within them (Johnson and Buller, 2011). However, research into host–pathogen interactions remains dominated by the study of one host–one pathogen systems despite the fact that hosts can simultaneously carry several infectious agents, with consequences for the dynamics of each agent and host health. Coinfections have been previously reported in shellfish hosts: including *Vibrio tapetis*, *Perkinsus* sp. and digenetic trematodes in cockles (Lassalle *et al.*, 2007); ostreid herpesvirus-1 (OsHV-1) and *Vibrio* spp. in oysters (Petton *et al.*, 2015; Alfaro *et al.*, 2019); and protozoans and bacteria in clams (Arzul *et al.*, 2012; Carella *et al.*, 2020); and in some of the cases with a dramatic effect on disease susceptibility (Cox, 2001; Lassalle *et al.*, 2007; Arzul *et al.*, 2012; Alfaro *et al.*, 2019). Processes at different scales of ecological organization from the within-host level (e.g. interactions of coinfecting parasites) to the ecosystem level (e.g. the influence of environmental variables) across space and over time have a key influence in complex natural systems (Hellard *et al.*, 2015). Therefore, moving from one host–one pathogen systems towards an ecosystem view of host–pathogen interactions and their ecology is essential.

Detection of interspecific parasite/pathogen interactions in natural populations is not easy, due to complex networks of indirect effects making it difficult to infer underlying processes (Hellard *et al.*, 2012). Interactions among coinfecting infectious agents can, in fact, alter host pathology, parasite transmission and virulence evolution, also influencing the spread of infections at a population level resulting in disease outbreaks, as described previously in bivalves (Lassalle *et al.*, 2007; Arzul *et al.*, 2012; Alfaro *et al.*, 2019). These effects can be the opposite, i.e. coinfections within the host may reduce infection success yet still enhance pathology or, in contrast, co-occurring parasites/pathogens may weaken host pathology increasing the infection success (Johnson and Hoverman, 2012). For example, mortality induced by one parasite can also eliminate the availability of hosts for other parasites (Jolles *et al.*, 2008); likewise, morbidity induced by one parasite can increase exposure to a second, even if within-host interactions are antagonistic (Karvonen *et al.*, 2009).

Simultaneous infections may therefore trigger a whole spectrum of outcomes within the hosts, both synergistic and antagonistic, i.e. the weight of one or both infectious agents may be suppressed (Fukami *et al.*, 2010), one or both agents may be amplified resulting in coexistence (Thomas *et al.*, 1998), or one may be amplified and the other suppressed (Johnson and Hoverman, 2012). Parasite/pathogen interactions can be not only direct, such as competition for attachment sites, competition for host resources and predation upon one another (intraguild predation) (Hatcher *et al.*, 2006, 2012; Johnson *et al.*, 2010), but interactions can also be indirect or host-mediated often involving changes in immunity such as cross-immunity

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(apparent competition) and immune suppression (apparent facilitation) (Jolles *et al.*, 2008). For instance, Johnson and Hoverman (2012) suggested cross-reactive immunity as a cause of a decrease in parasite persistence in their experiments with a group of related larval trematodes in Pacific chorus frogs (*Pseudacris regilla*). Johansen and Sommer (2001) suggested that the immune suppression triggered by a primary infection could be the cause of multiple bacterial infections in Atlantic salmon (*Salmo salar*).

The co-occurrence of infectious agents may also result simply because the same risk factors promote their presence and not because the different pathogen groups are interacting synergistically (Vaumourin *et al.*, 2015). Risk factors shared by two or more infectious agents can occur at the level of the host individual (e.g. host condition and age), population (e.g. host density) or landscape (e.g. climatic factors), increasing the probability of infection by both/all agents (Hellard *et al.*, 2015). Such confounding factors may influence host exposure and/or host susceptibility promoting simultaneous infections (Vaumourin *et al.*, 2015). Environmental parameters such as seawater temperature and salinity, host density or host physiological conditions and behaviour are common confounding factors that may influence the co-occurrence of different pathogen groups and the epidemiological and geographic patterns of infection and disease (Hellard *et al.*, 2012; Vaumourin *et al.*, 2015). Confounding factors must be considered in field studies since those factors can create statistical associations between infectious agents even if there is no true biological interaction between them (Kuris and Lafferty, 1994).

Generalist host species, such as the common cockle *Cerastoderma edule*, with wide spatial distributions, are exposed to a large range of environmental conditions, and a greater diversity of parasites and pathogens, and consequently are more likely to be coinfected (Vaumourin *et al.*, 2015; Mahony *et al.*, 2020; de Montaudouin *et al.*, 2021), 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.*, 2010, 2021), but may also be infected by a range of pathogens (Rowley *et al.*, 2014; de Montaudouin *et al.*, 2021), providing a microcosm for the parasite/pathogen community. Within this community, there are haplosporidian protists such as the genus *Minchinia* (Engelsma *et al.*, 2011; Longshaw and Malham, 2013; Ramilo *et al.*, 2018; Lynch *et al.*, 2020*b*; Albuixech-Martí *et al.*, 2020; de Montaudouin *et al.*, 2021), numerous members of the bacterial family *Vibrio* (Carballal *et al.*, 2001; Lassalle *et al.*, 2007; de Montaudouin *et al.*, 2021), viruses such as ostreid herpesvirus-1 microVar (OsHV-1 μVar) (Carballal *et al.*, 2003; Evans *et al.*, 2017; Bookelaar *et al.*, 2020; de Montaudouin *et al.*, 2021) and microsporidian species reported in digeneans and paramyxids infecting cockle populations (Goater, 1993; Fermer *et al.*, 2009, 2011; Villalba *et al.*, 2014; de Montaudouin *et al.*, 2021). Cockles are a keystone species responsible for different ecosystem services, i.e. carbon storage, energy cycling, food source for seabirds, etc., being also good sentinel and bioindicator species (Malham *et al.*, 2012; Carss *et al.*, 2020). Moreover, the common cockle is one of the main non-cultured bivalve species harvested in western European waters, with a high economic value (Rowley *et al.*, 2014; Carss *et al.*, 2020). Consequently, cockles have been well-studied and are useful model species. Given the complex parasite/pathogen assemblages within the cockles and their intricate interactions and effects, a comprehensive and integrated approach that considers not only the synergistic impact of coinfections but also the role of ecological factors driving the infection across space and over time is needed.

Future climate scenarios predict a warming marine environment and increased precipitation events resulting in pulse events of reduced/fluctuating salinity in nearshore ecosystems (Allam and Raftos, 2015; Coen and Bishop, 2015). Consequently, climate change may be expected to have a significant effect on disease epidemiology (Coen and Bishop, 2015), and may even have significant consequences on zoonoses emergence (Hoarau *et al.*, 2020). In this context, the need for the understanding of ecological processes involved in the transmission and dynamics of infectious agents in host reservoirs is critical (Hoarau *et al.*, 2020). This study, therefore, aims to assess the interactions between infectious agents, their hosts and the environment.

For that purpose, this study examined the diversity and prevalence of significant pathogen groups, Haplosporidia, *Vibrio* spp., OsHV-1 μVar and Microsporidia, associated with bivalve mortalities globally as well as coinfections and associations of the different infectious agents at an individual and population level in wild and fished *C. edule* along the Irish and Celtic Seas. The role of abiotic (seawater temperature and salinity) and biotic (cockle size and age, and epiflora on their shells) factors in the risk of coinfection throughout the study was also assessed. Developing knowledge on pathogen diversity and co-occurrence in an economically and ecologically important species as is *C. edule* and integrating the role of the environment in the host–pathogen interplay is essential for a better understanding of the interaction networks that can affect disease outcomes and transmissibility in the current changing environment. Findings from this study highlight the complexity of pathogen interactions within a host and the effect of key biotic and environmental drivers shaping disease dynamics, which can also be applied to other coinfected animal host species.

Materials and methods

Sampling

Cockles were sampled from spring 2018 to spring 2019 at Cork Harbour (Ringaskiddy and Cuskinny, $n = 257$) on the south coast (Celtic Sea) of Ireland and from summer 2018 to spring 2019 at Youghal Bay ($n = 69$) and Dungarvan Harbour ($n = 169$), with an extensive farming of Pacific oysters (*Crassostrea gigas*), on the south coast (Celtic Sea) of Ireland, where there is no commercial fishing activity (Fig. 1). Additionally, 240 cockles were collected from summer 2018 to spring 2019 at the commercial fishery at Dundalk Bay (Annagassan and Cooley) on the northeast coast (Irish Sea) of Ireland (Fig. 1). Dundalk Bay is a classified Bivalve Mollusc Production Area and it has supported a commercial dredge cockle (*C. edule*) fishery since 2001 (Hervas *et al.*, 2008).

The four locations are bays with intertidal sand and mudflats, where a variety of bivalves inhabit. *Cerastoderma edule* densities in the selected sites vary from higher densities, 9.33 ± 3.5 ind m^{-2}, in Dundalk Bay (Shellfish Stocks and Fisheries Review, Marine Institute and BIM, 2017) to lower densities in the no commercial fishing sites: 1.6 ± 2.1 ind m^{-2} in Youghal Bay; 0.8 ± 1.7 ind m^{-2} in Cork Harbour; and 0.4 ± 1.3 ind m^{-2} in Dungarvan Harbour (Fermer *et al.*, 2011). Cockle mortalities associated with disseminated neoplasia have been previously reported in Cork Harbour (Morgan *et al.*, 2012). While a massive cockle mortality event associated with dinoflagellates occurred in Youghal Bay in the past (Ottway *et al.*, 1979). Dungarvan Harbour has also a history of OsHV-1-associated mortality events (Lynch *et al.*, 2012; Prado-Alvarez *et al.*, 2016). However, to the best of our knowledge, Dundalk Bay has not previously recorded mortality events.

Even though Cork Harbour is an important industrial area and is a busy ferry/shipping terminal, its trophic status (based on the nutrient levels such as phosphorus and nitrogen, growth of algae and dissolved oxygen concentration) was qualified as unpolluted by the EPA in 2017 along with Dungarvan Harbour, an extremely sheltered bay, while Youghal Bay, close to an urban hub, was

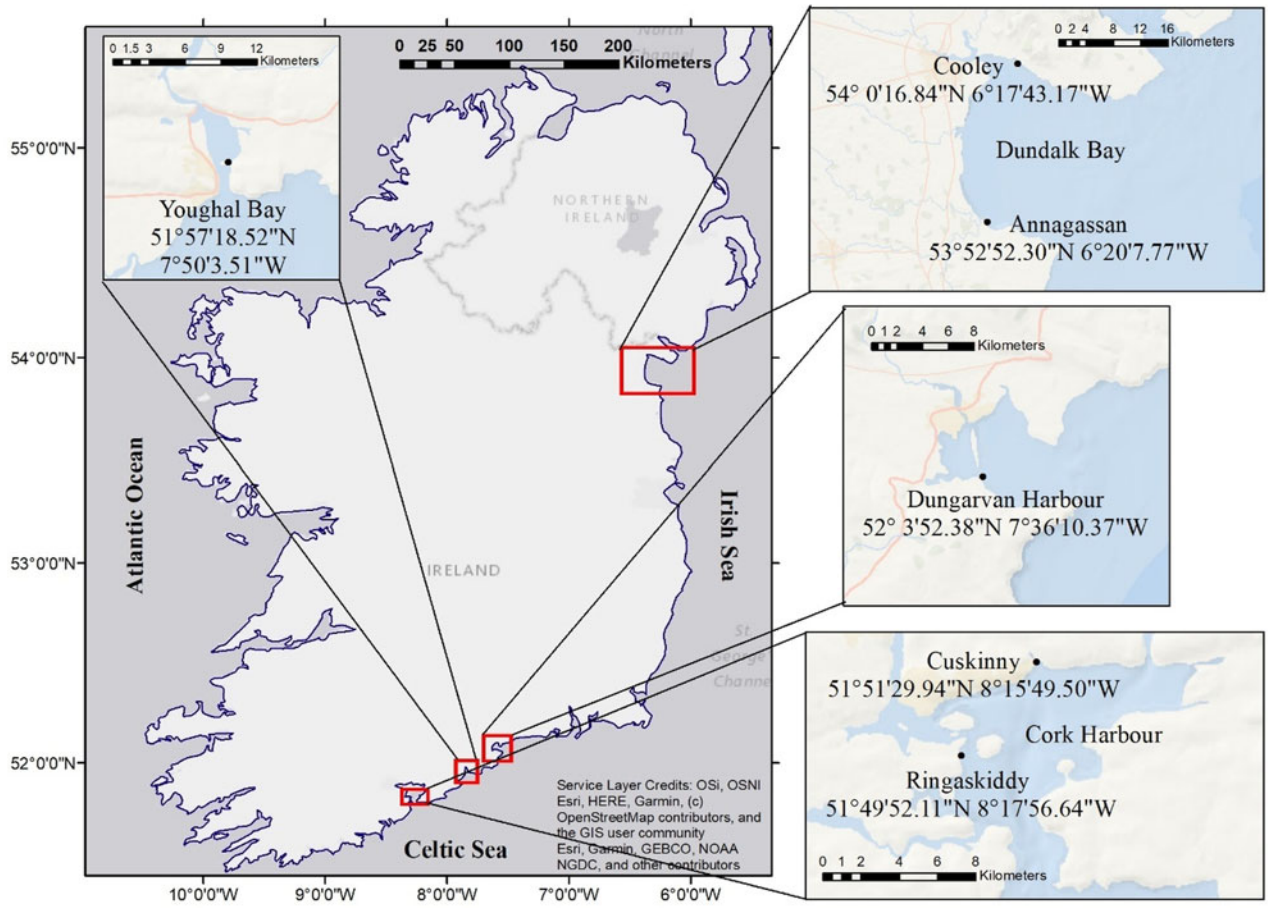

Fig. 1. Map of Ireland highlighting the cockle sample sites with coordinates.

classified as intermediate polluted area. In turn, Dundalk Bay showed a localized effect on the trophic status, with Annagassan classified as an unpolluted area and Cooley as an intermediate polluted area, probably due to the influence of water discharge from the local river.

Cockles were collected by hand raking from the intertidal area of each site at spring tide (0.5–1 m) and seasonal intervals. The sample size, outlined in Table 1, was dependant on the availability of cockles at each site and season. Cockles were kept at ambient temperature during the fieldwork and were processed either on the same day or were held out of water overnight at 4 °C and were processed the next day.

Sample processing and diagnostic methods

Morphometrics

Measurements included (A) cockle body size [height (mm)] of each individual that was recorded using Vernier callipers. The (B) cockle's age was established 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 recognized that variations in growth during the same year lead to the possible formation of extra stria (de Montaudouin, 1996). Ultimately, the presence or absence of epiflora on the shell of each individual was also recorded. Epiflora was mainly composed of epiphytic algae, although no specific species were identified.

Histology

A transverse section of each cockle was excised, including the digestive gland, gonads, gills and mantle tissues; as well as a section of the hinge ligament and a section of the tip of the foot were taken 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 5 μM and stained using haematoxylin and eosin (Sigma Aldrich, USA) (Howard and Smith, 1983). Tissue slides were screened at a magnification of 40x and under oil at 100x for visualization of the pathogens and tissue pathology.

Molecular techniques for cockle species identification and pathological 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 European cockle species [*C. edule* (Linnaeus, 1758) or *Cerastoderma glaucum* (Poiret, 1789)] and for subsequent pathological screening. Genomic DNA from the cockle gill tissue was extracted using the chelex-100 extraction method (Walsh *et al.*, 1991; Lynch *et al.*, 2008).

Polymerase chain reaction (PCR) was carried out to amplify the nuclear DNA markers *ITS-for*/*ITS Ce-R*/*ITS Cg-R* to differentiate between the presence of *C. edule*, *C. glaucum* species or hybrids (Freire *et al.*, 2011; Table 2). Amplification was conducted following the reaction mixture and thermocycling conditions, as well as the visualization of the product, described in Albuixech-Martí *et al.* (2020).

Standard PCR screening for haplosporidian detection was carried out in cockle gill tissue samples ($n = 735$) 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 2). Amplification was

Table 1. Sample sites, seasons and number of cockles collected

Sampling dates	Cork Harbour	Youghal Bay	Dungarvan Harbour	Dundalk Bay	Totals
Spring 2018	60	–	–	–	60
Summer 2018	39	11	51	60	161
Autumn 2018	60	16	58	60	194
Winter 2018/19	38	30	30	60	158
Spring 2019	60	12	30	60	162
Totals[a]	257 (34.9%)	69 (9.4%)	169 (23.0%)	240 (32.7%)	735

(–) No samples collected.
[a]In brackets the % representation of all cockles sampled.

Table 2. Description of PCR primer pairs showing sequences for each forward and reverse primer and expected product size

	Primer sequence (5′–3′)			
Primer pair	Forward	Reverse	Product size (bp)	Primer specificity
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	350	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
Vib-F3 Vib-R3	CAA CAG AAG AAG CAC CGG CT	CAC GCT TTC GCA TCT GAG TG	286	*Vibrio* spp.
OHVA OHVB	TGC TGG CTG ATG TGA TGG CTT TGG	GGA TAT GGA GCT GCG GCG CT	385	*Ostreid herpesvirus-1 and variants*
MicIF1 MicIR1	GTG GAC GCT AGT CTC ACA GGT T	TTG CAC CAG AAG GTT TAC GAC ACA T	180	*Microsporidia sp.1*
MicIF2 MicIR2	ATG CAT GCG TAA GCG AAG CAG TTA T	TCT CTT GCA CCA GAA GGT TTA CGA C	180	*Microsporidia sp. 2*

conducted following the reaction mixture and thermocycling conditions, as well as the visualization of the product, described in Albuixech-Martí *et al.* (2020).

Standard PCR screening for *Minchinia* spp. detection was carried out in the cockle samples that were positive for Haplosporidia by conventional PCR, using specific primers for *Minchinia tapetis* (TAP-For/Rev) and *Minchinia mercenariae*-like (MER-For/Rev) (Albuixech-Martí *et al.*, 2020; Table 2) in the appropriate pairings and separate PCR reactions. Amplification was conducted following the reaction mixture and thermocycling conditions, as well as the visualization of the product, described in Albuixech-Martí *et al.* (2020).

Standard PCR screening for *Vibrio* spp. in cockle gill tissues was conducted using generic primers Vib-F3/R3, developed in our lab for the detection of *Vibrio* genus [Kett *et al.*, unpublished as per Notaro *et al.* (2021), data confirmed to be specific for *Vibrio* spp. by Sanger sequencing; Table 2]. The screening was conducted in all the collected cockles ($n = 735$). A total of 2 μL of DNA per individual was used in a total volume of 27.5 μL of the reaction mixture containing: 5 μL of 5x green buffer, 5 μL of dNTPs (1.25 mM), 0.5 μL of $MgCl_2$, 0.25 μL of forward and reverse primers, 0.1 μL of GoTaq polymerases, 1.5 μL of DMSO and 12.9 μL of ddH_2O. Cycling conditions consisted of an initial denaturation of the sample at 95 °C for 1 min followed by 35 cycles of 94 °C for 20 s, 56 °C for 30 s, 72 °C for 30 s and a final elongation at 72 °C for 7 min. Electrophoresis of the amplification products was conducted in a 2% agarose gel.

Standard PCR screening for OsHV-1 and variants, including OsHV-1 μVar, was carried out in cockle gill tissue samples ($n = 735$) using specific primers OHVA/OHVB that amplify small products of the *ORF4* gene in the OsHV-1 virus and its variants (Lynch *et al.*, 2013; Table 2). Amplification was conducted following the reaction mixture and thermocycling conditions described in Lynch *et al.* (2013). Electrophoresis of the amplification products was conducted in a 2% agarose gel.

Standard PCR using specific primers MicIF1/MicIR1 and MicIF2/MicIR2 (Lynch *et al.*, unpublished data confirmed to be specific to Microsporidia spp. by Sanger sequencing; Table 2) to detect two unidentified microsporidian species was carried out in cockle gill tissue samples ($n = 735$). Both reaction mixture and thermocycling conditions were the same as used with the PCR for OsHV-1 and variants, as well as the visualization of the product.

Real-time quantitative PCR (qPCR) for detection and quantification of *Vibrio aestuarianus* in the cockle samples that were positive for *Vibrio* spp. was performed using specific primers (Table 3) for detection of the molecular chaperone *dnaJ* gene,

Table 3. Description of qPCR primer pairs and probe

Primers	Sequence	Specificity
dnaJ f420	GTGAAGGGACGGGTGCTAAG	*Vibrio aestuarianus*
dnaJ r456	CCATGACAAGTGCCACAAGTCT	
dnaJ p441 (probe)	FAM-AGGGCACGTCGGC-MGB	
HVDP-F	ATTGATGATGTGGATAATCTGTG	*Ostreid herpesvirus-1 and variants*
HVDP-R	GGTAAATACCATTGGTCTTGTTCC	

following the protocol of McCleary and Henshilwood (2015). All samples were performed in triplicates using 5 μL of DNA. C_t values were used to determine real-time PCR quantification and detection limits. A tested sample was considered positive if its mean C_t value was below 37.

Viral load for OsHV-1 and variants was investigated by qPCR with a small number of individuals ($n = 30$, whose standard PCR results displayed some smear in the visualization of the product) to verify that there was no viral load by carrying out a more specific screening and increasing the amount of DNA processed. The protocol of Pepin *et al.* (2008) was followed, using the specific primers HVDP-F/HVDP-R (Table 3). qPCR was conducted in duplicate using 5 μL DNA by a Thermo Hybaid PCR express thermal cycler.

Direct sequencing was carried out on representative PCR products ($n = 10$) amplified using generic *Vibrio* Vib-F3/R3 primers (Kett *et al.*, unpublished as per Notaro *et al.*, 2021) to identify the *Vibrio* species present in the samples. Likewise, direct sequencing was carried out on representative PCR products ($n = 5$) 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 beyond the *Minchinia* species screened. Genomic DNA from selected individuals 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 (Source Bioscience). Each sequence was matched against the National Center for Biotechnology Information (NCBI) nucleotide database with BLASTn (Basic Local Alignment Search Tool), which finds regions of local similarity between sequences to identify and confirm the DNA being detected in the PCRs.

Environmental data

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

Statistical analysis

Statistical analysis was performed in R version 3.2.3. statistical software. The pathogen occurrence was modelled as pathogen presence/absence in each cockle for each pathogen group and their combinations, and it was used in the subsequent analysis to examine the associations between the different pathogen groups present through the samples.

Association screening approach (SCN), described in detail by Vaumourin *et al.* (2014), was conducted running 5000 simulations (NS) with the presence/absence of Haplosporidia and *Vibrio* spp. to determine a potential association between these two pathogen groups, under the null hypothesis of random pathogen associations [$P(\text{SCN}) < 0.05$]. Subsequently, a second SCN (NS = 5000) was performed with the presence/absence of the different pathogen species detected and quantified in the samples, i.e. *M. tapetis*, *M. mercenariae*-like and *V. aestuarianus*.

Corrected Pearson's χ^2 test, described in detail by Hellard *et al.* (2012), was applied to the Haplosporidia and *Vibrio* spp. presence/absence data to determine if the coinfection was due to an underlying biological interaction or to confounding factors considered (seawater temperature, salinity, cockle size and age, and epiflora on their shells). Likewise, the test was applied to the *M. tapetis* and *M. mercenariae*-like presence/absence data. Biological interactions between two pathogens, therefore, will be suspected when the probability of coinfection is not random once confounding factors have been considered [$P(\text{Corr } \chi^2) < 0.05$]. In the model, running 1000 bootstraps, the effects of the confounding factors are summarized as F1 + F2 + F3 + F4 + F5.

Moreover, Pearson's χ^2 tests were used to determine whether the prevalence observed of single infections and two-pathogen coinfections was significantly different [$P(\text{Chisq}) < 0.05$] among the sample sites and throughout the year. Fisher's exact test was conducted when the frequencies of the pathogen occurrence in a site or season were <4, to gain accuracy.

Results

In total, 735 cockles were screened to determine the prevalence of infection and coinfections of the target pathogens (Table 4). The cockle speciation PCR confirmed that all the individuals from all sample sites were *C. edule*.

Diversity and prevalence of single and multiple infections and the associations between them

No OsHV-1 μVar nor microsporidian species were detected in any of the cockles ($n = 735$) screened by PCR. The only pathogen groups detected during the study were Haplosporidia and *Vibrio*. Overall, Haplosporidia was the most common pathogen found through the samples, with a 37.7% ($n = 277$) occurrence (Fig. 2, detailed data in Table 4). Among these 277 infected cockles, two haplosporidian species were identified in the study: *M. tapetis* which was present in 29.6% ($n = 82/277$) of the tested individuals, and *M. mercenariae*-like which was present in 14.8% ($n = 41/277$) (Fig. 2, detailed data in 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 for *Minchinia* spp., which could indicate the presence of other haplosporidian species occurring in the samples. Thus, five of those samples amplified using generic HAP-F1/R3 primers were commercially sequenced; however, low-complexity sequences were obtained, and no significant similarity was found in GenBank. Therefore,

Table 4. Prevalence (%, positive individuals/screened individuals) of Haplosporidia spp., *Minchinia tapetis*, *M. mercenariae*-like, *Vibrio* spp. and *Vibrio aestuarianus* screening in *Cerastoderma edule* 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 and Cuskinny	Haplosporidia spp.	**45.0% (27/60)**[a]	15.4% (6/39)	41.7% (25/60)	21.1% (8/38)	28.3% (17/60)	32.3% (83/257)
	Minchinia tapetis	15.0% (9/60)	7.7% (3/39)	**18.3% (11/60)**[a]	2.6% (1/38)	1.7% (1/60)	9.7% (25/257)
	Minchinia mercenariae-like	**23.3% (14/60)**[a]	0% (0/39)	8.3% (5/60)	0% (0/38)	10.0% (6/60)	9.7% (25/257)
	Vibrio spp.[b]	13.3% (8/60)	12.8% (5/39)	**15.0% (9/60)**[a]	5.3% (2/38)	11.7% (7/60)	12.1% (31/257)
	V. aestuarianus	**1.7% (1/60)**[a]	0% (0/39)	0% (0/60)	0% (0/38)	**1.7% (1/60)**[a]	0.8% (2/257)
Youghal Bay	Haplosporidia spp.	–	9.1% (1/11)	12.5% (2/16)	**56.7% (17/30)**[a]	8.33% (1/12)	30.4% (21/69)
	Minchinia tapetis	–	0% (0/11)	0% (0/16)	0% (0/30)	0% (0/12)	0% (0/69)
	Minchinia mercenariae-like	–	0% (0/11)	**12.5% (2/16)**[a]	3.3% (1/30)	0% (0/12)	4.3% (3/69)
	Vibrio spp.[b]	–	27.3% (3/11)	**43.8% (7/16)**[a]	16.7% (5/30)	33.3% (4/12)	27.5% (19/69)
	V. aestuarianus	–	**27.3% (3/11)**[a]	6.3% (1/16)	0% (0/30)	0% (0/12)	5.8% (4/69)
Dungarvan Harbour	Haplosporidia spp.	–	**39.2% (20/51)**[a]	10.3% (6/58)	30% (9/30)	40% (12/30)	27.8% (47/169)
	Minchinia tapetis	–	**2.0% (1/51)**[a]	0% (0/58)	0% (0/30)	0% (0/30)	0.6% (1/169)
	Minchinia mercenariae-like	–	0% (0/51)	0% (0/58)	0% (0/30)	**10.0% (3/30)**[a]	1.8% (3/169)
	Vibrio spp.[b]	–	**56.9% (29/51)**[a]	**56.9% (33/58)**[a]	6.7% (2/30)	36.7% (11/30)	44.4% (75/169)
	V. aestuarianus	–	**43.1% (22/51)**[a]	0% (0/58)	0% (0/30)	3.3% (1/30)	13.6% (23/169)
Dundalk Bay – Annagassan and Cooley	Haplosporidia spp.	–	**98.3% (59/60)**[a]	28.3% (17/60)	45.0% (27/60)	38.3% (23/60)	52.5% (126/240)
	Minchinia tapetis	–	**70.0% (42/60)**[a]	23.3% (14/60)	0% (0/60)	0% (0/60)	23.3% (56/240)
	Minchinia mercenariae-like	–	**11.7% (7/60)**[a]	0% (0/60)	1.7% (1/60)	3.3% (2/60)	4.2% (10/240)
	Vibrio spp.[b]	–	**38.3% (23/60)**[a]	33.3% (20/60)	3.3% (2/60)	26.7% (16/60)	25.4% (61/240)
	V. aestuarianus	–	3.3% (2/60)	**10% (6/60)**[a]	0% (0/60)	0% (0/60)	3.3% (8/240)
Totals by season	Haplosporidia spp.	45.0% (27/60)	**53.4% (86/161)**[a]	25.8% (50/194)	38.6% (61/158)	32.7% (53/162)	37.7% (277/735)
	Minchinia tapetis	15.0% (9/60)	**28.6% (46/161)**[a]	12.9% (25/194)	0.6% (1/158)	0.6% (1/162)	11.2% (82/735)
	Minchinia mercenariae-like	**23.3% (14/60)**[a]	4.3% (7/161)	3.6% (7/194)	1.3% (2/158)	6.8% (11/162)	5.6% (41/735)
	Vibrio spp.[b]	13.3% (8/60)	**37.3% (60/161)**[a]	35.6% (69/194)	7.0% (11/158)	23.5% (38/162)	25.3% (186/735)
	V. aestuarianus	1.7% (1/60)	**16.8% (27/161)**[a]	3.6% (7/194)	0.0% (0/158)	1.2% (2/162)	5.0% (37/735)

(–) No samples.
[a]The highest prevalence (%) for each pathogen group at each site/season is highlighted in bold.
[b]Potential *Vibrio* species were defined in Table 5.

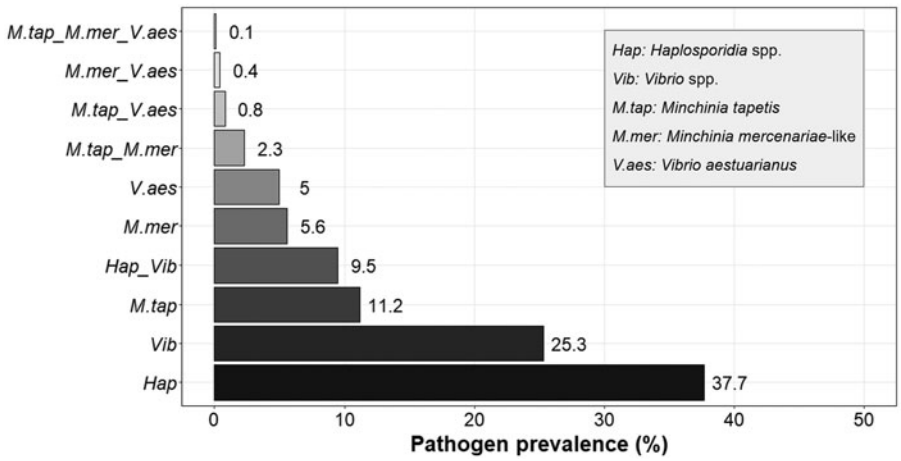

Fig. 2. Overall prevalence of infections and coinfections (%) with the different pathogen groups (Hap: Haplosporidia spp.; Vib: *Vibrio* spp.; M.tap: *Minchinia tapetis*; M.mer: *Minchinia mercenariae*-like; V.aes: *Vibrio aestuarianus*).

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

Vibrio was present in 25.3% ($n = 186$) of the total number of cockles screened (Fig. 2, detailed data in Table 4). Of the 186 samples that were positive for *Vibrio*, 19.9% ($n = 37/186$) were confirmed by qPCR to be *V. aestuarianus* (Fig. 2, detailed data in Table 4). *Vibrio aestuarianus*-positive controls (diluted purified DNA) have a $C_t = 30$ (60 copies of *V. aestuarianus* DNA per μL). Based on that value, 13.5% ($n = 5/37$) of the individuals were classified as heavily infected individuals ($C_t \leqslant 30$), while 86.5% ($n = 32/37$) were classified as low-intensity infections ($37 > C_t > 30$).

Direct sequencing was carried out on representative PCR products ($n = 10$) amplified using generic *Vibrio* Vib-F3/R3 primers (Kett *et al.*, unpublished as per Notaro *et al.*, 2021) to identify other *Vibrio* species present in the samples apart from *V. aestuarianus*. Using BLASTn, three of the sequenced samples had a query length of 127–155 bp with an 83–91% query coverage and 92.6–97.9% identity to *Vibrio splendidus* deposited by Gao (2020) (MT445179.1; MT445177.1) and by Landreau *et al.* (2020) (MT345091.1) (Table 5). Three other PCR product samples that generated sequences had a query length of 82–179 bp, a query coverage of 78–83% and 92.6–98.6% identity to *Vibrio kanaloae* deposited by Zheng (2020) (MT505700.1) and by Barcia and Romalde (2020) (CP065151.1; CP065150.1) (Table 5). Another sample, with a query length of 158 bp, had 94% query coverage and 98% identity to *V. aestuarianus* deposited by Garcia (2018) (MK307696.1) and *Vibrio mediterranei* deposited by Gao *et al.* (2020) (MT269602.1) (Table 5). Two other samples were identified as *Vibrio* spp., as the blasted sequence was quite short (52–69 bp) to differentiate robustly between species (Table 5). The 186 positive PCR cases were defined as *Vibrio* spp. for statistical and analysis purposes.

Overall, 9.5% ($n = 70/735$) of the individuals displayed coinfection with the two pathogen groups present in the samples, Haplosporidia and *Vibrio* (Fig. 2). However, no significant association between them was displayed when testing by SCN (P value > 0.05), under the null hypothesis of random pathogen combinations (Table 6), i.e. the pathogen combination was considered not to occur differently than expected by chance.

In order to identify the mechanisms driving the pathogen coinfection with Haplosporidia and *Vibrio* pathogen groups, corrected Pearson's χ^2 test was also performed including seawater temperature, salinity, cockle height and age, as well as the presence/absence of epiflora on the shells (Tables 7 and 8), as confounding factors which may promote pathogen presence. Under the null hypothesis of independence between the two pathogens, the test showed that the observed frequencies for each pathogen combination were not significantly different (Corr $\chi^2 = 2.51$; $\hat{c} = 0.84$; P value 1 = 0.08; P value 2 = 0.08) to the expected frequencies considering the confounding factors as drivers of the infection (Supplementary material). Therefore, the proportion of double infected individuals can be explained because the same factors promote the pathogen presence and not because the different pathogen groups are interacting synergistically. The differences in the considered environmental and biotic factors between sample sites and throughout the time, thus, might have been driving the risk of infection in *C. edule* populations.

Regarding the specific species quantified in the samples, only 0.8% ($n = 6/735$) of the individuals were coinfected with *V. aestuarianus* and *M. tapetis*, while 0.4% ($n = 3/735$) were coinfected with *V. aestuarianus* and *M. mercenariae*-like (Fig. 2). Likewise, 13.8% of the *Minchinia*-infected cockles showed a coinfection with both *Minchinia* species ($n = 17/123$); however, the percentage fell to 2.3% when all the screened cockles were considered ($n = 17/735$) (Fig. 2). While coinfection with both *Minchinia* species and *V. aestuarianus* was only observed in one of the samples (0.1%, $n = 1/735$) (Fig. 2).

Associations between *M. tapetis*, *M. mercenariae*-like and *V. aestuarianus* and all the possible combinations were tested by SCN, revealing a significant association between the two species of *Minchinia* (Table 9). The observed number of coinfected individuals with both *Minchinia* species were significantly overrepresented compared to expected by random chance (P value < 0.001; Table 9), indicating a significant positive two-way association between these two species. While *M. mercenariae*-like was found to occur alone significantly less often than expected by chance (P value < 0.05; Table 9).

The association between *Minchinia* species was further examined by corrected Pearson's χ^2 test in order to assess if the biotic and abiotic factors considered in the study (Tables 7 and 8) influenced this coinfection. The test showed that the observed frequencies for each pathogen combination were significantly different from the expected frequencies considering the confounding factors as drivers of the infection (Supplementary material). The significant outcome (Corr $\chi^2 = 3.04$; $\hat{c} = 0.59$; P value 1 = 0.02; P value 2 = 0.02) showed that both pathogens are not independent, and the proportion of double infected individuals cannot be due only to shared confounding factors.

Site influence on single infections and coinfections

Spatial variability was assessed and significant differences [P(Chisq) < 0.05] were found between the sample sites when the prevalence of single infections and two-pathogen coinfections was considered (Fig. 3). Dundalk Bay was the site with the highest presence of Haploporidia spp. (52.5%, $n = 126/240$), showing a

Table 5. Description of the BLASTn results obtained from the sequenced DNA of cockle samples using generic *Vibrio* primers Vib-F3/R3

Sample site	Season	Species identification	Per cent identity	Query cover	Query length (bp)
Youghal Dungarvan Cooley	Summer 2018	*Vibrio spp.* among them: *Vibrio splendidus* and *V. kanaloae*	92.59–98.58%	78–83%	82–179
Annagassan Annagassan	Summer 2018	*Vibrio spp*. most likely *V. splendidus*	97.84–97.92%	88–91%	154–155
Annagassan	Summer 2018	*Vibrio spp.* most likely *V. aestuarianus* or *V. mediterranei*	98.01%	94%	158
Cuskinny Dungarvan	Autumn 2018	*Vibrio spp.*	94.23–94.29%	67–73%	52–69

Table 6. Association screening analysis (SCN) results for all the pathogen combinations between Haplosporidia (HAP) and *Vibrio* (VIB) pathogen groups

Coinfection status	Pathogen groups		Obs	LL	UL	SCN *P* value
2-pathogen coinfection	HAP	VIB	70	51	90	0.5174
1-pathogen infection	HAP		207	177	236	0.9832
		VIB	116	92	141	0.5142
Not infected			342	309	376	0.5222

The observed (Obs) frequency of each coinfection status is given along with the lower (LL) and upper (UL) limits of the 95% confidence envelope.

Table 7. Mean ± S.D. of abiotic (seawater temperature and salinity) and biotic factors (cockle height and age) and the overall percentage of the presence of epiflora on the cockle shells at each sample site

Mean ± S.D.	Cork Harbour	Youghal Bay	Dungarvan Harbour	Dundalk Bay
Temperature (°C)	11.8 ± 2.6	10.7 ± 2.9	**12.2 ± 3.0**	10.8 ± 4.2
Salinity (PSU)	33.6 ± 0.8	**33.9 ± 0.03**	**33.9 ± 0.2**	30.4 ± 0.5
Height (mm)	**29.7 ± 7.8**	27.3 ± 4.8	22.0 ± 4.5	29.6 ± 5.3
Age (growth rings)	**3.6 ± 1.9**	2.9 ± 1.7	1.7 ± 1.0	3.0 ± 1.6
Epiflora (presence/absence)	**10.9%**	7.2%	4.7%	1.7%

The largest value for each factor is highlighted in bold.

Table 8. Mean ± S.D. of abiotic (seawater temperature and salinity) and biotic factors (cockle height and age) and the overall percentage of the presence of epiflora on the cockle shells by season

Mean ± S.D.	Spring 2018	Summer 2018	Autumn 2018	Winter 2018/19	Spring 2019
Temperature (°C)	12.2 ± 3.9	12.0 ± 3.3	11.9 ± 3.3	11.5 ± 3.4	11.4 ± 3.4
Salinity (PSU)	32.2 ± 1.6	33.1 ± 1.4	32.9 ± 1.6	32.7 ± 1.7	32.6 ± 1.7
Height (mm)	28.0 ± 7.4	27.2 ± 7.1	27.5 ± 6.9	27.8 ± 6.7	27.9 ± 6.6
Age (growth rings)	2.7 ± 1.7	2.8 ± 1.7	2.8 ± 1.8	2.9 ± 1.8	2.9 ± 1.7
Epiflora (presence/absence)	26.7%	8.7%	2.1%	1.9%	4.9%

substantially higher prevalence of *M. tapetis* (23.3%, $n = 56/240$) than the other sites, while no presence of *M. tapetis* was observed in Youghal Bay. Dundalk Bay showed lower seawater salinity and a lower presence of epiflora on the cockle shells than the other sample sites (Table 7). Dungarvan Harbour, with the smallest and youngest cockles collected (Table 7), showed the highest presence of *Vibrio* spp. (44.4%, $n = 75/169$) and a peak of *V. aestuarianus* (13.6%, $n = 23/169$). Moreover, three out of the five individuals with the highest intensity of infection ($C_t \leqslant 30$) with *V. aestuarianus* were observed at Dungarvan Harbour. Accordingly, the highest prevalence of coinfection with Haplosporidia and *Vibrio* spp. occurred in Dundalk Bay (14.6%, $n = 35/240$) and Dungarvan Harbour (11.8%, $n = 20/169$). However, coinfection with *M. tapetis* and *M. mercenariae*-like was absent in Dungarvan Harbour and Youghal Bay, with Cork Harbour being the site with the highest prevalence (4.3%, $n = 11/257$). The other two-pathogen coinfections with *Minchinia* species and *V. aestuarianus* showed no significant differences between the sites [P(Chisq) >0.05] since the total number of positive cases detected was too low [*M. tapetis* and *V. aestuarianus* ($n = 6$); *M. mercenariae* and *V. aestuarianus* ($n = 3$)]. Likewise, Cork Harbour was the only site that showed three-pathogen coinfection with both *Minchinia* species and *V. aestuarianus* (0.4%, $n = 1/257$). Cork Harbour, the site with the oldest

Table 9. Association screening analysis (SCN) results for all the pathogen combinations between *M. tapetis* (MT), *M. mercenariae*-like (MM) and *V. aestuarianus* (VA)

Coinfection status	Pathogen species			Obs	LL	UL	SCN *P* value
3-pathogen coinfection	MT	MM	VA	1	0	2	0.4292
2-pathogen coinfection	MT	MM		16	0	11	**0.0004**
	MT		VA	5	0	10	0.7036
		MM	VA	2	0	6	0.5568
1-pathogen infection	MT			60	53	95	0.0996
		MM		22	21	50	**0.0296**
			VA	29	18	46	0.8016
Not infected				600	558	612	0.1836

The observed (Obs) frequency of each coinfection status is given along with the lower (LL) and upper (UL) limits of the 95% confidence envelope. Significant associations (*P* value < 0.05) are highlighted in bold.

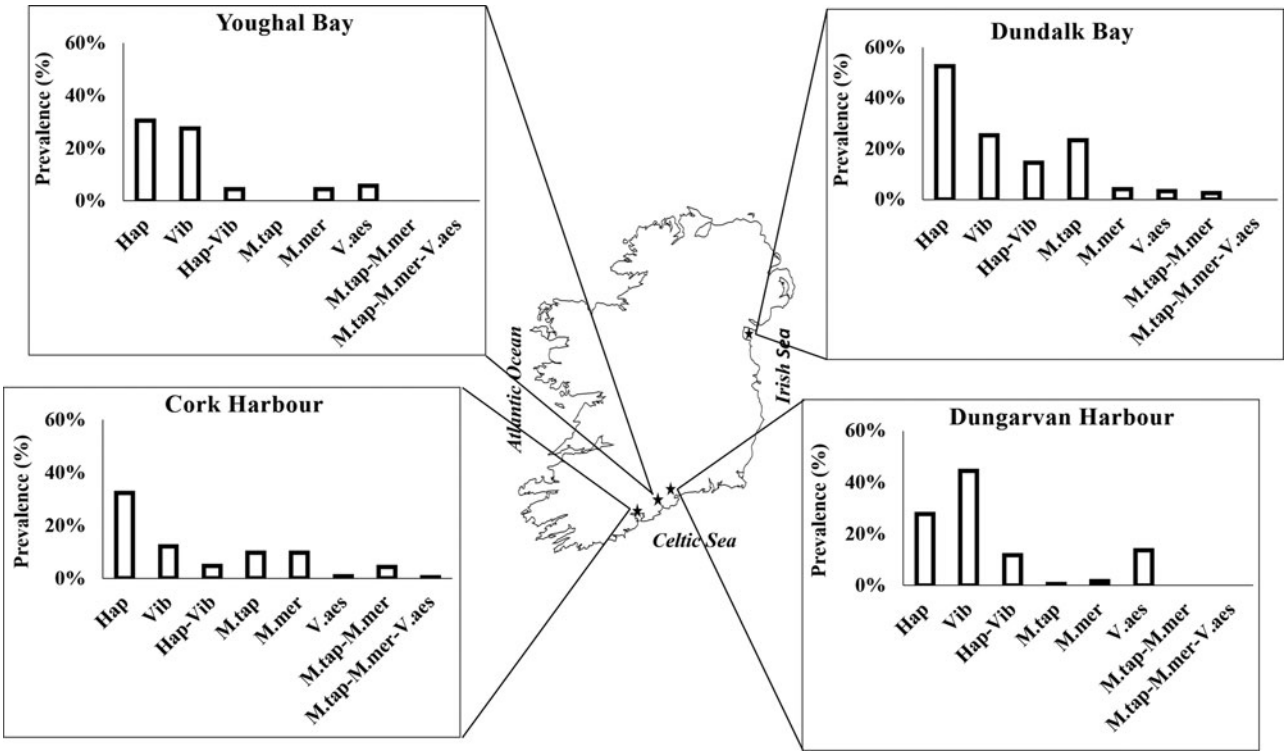

Fig. 3. Prevalence (%) of Haplosporidia spp. (Hap), *Vibrio* spp. (Vib), *Minchinia tapetis* (M.tap), *M. mercenariae*-like (M.mer) and *V. aestuarianus* (V.aes) single infections and coinfections (Hap_Vib; M.tap_M.mer; M.tap_M.mer_V.aes) at each sample site.

cockles, had the highest presence of epiflora on the cockle shells compared to the other sample sites (Table 7).

Seasonal impact on single infections and coinfections

Significant seasonal variability [P(Chisq) <0.001] was exhibited when the prevalence of single infections and two-pathogen coinfections were compared between seasons (Fig. 4). The absence of *V. aestuarianus* and, in general, a low prevalence of infections were observed in winter 2018/19 (Fig. 4), except for Haplosporidia spp. (38.6%, $n = 61/158$). Summer 2018 had the highest prevalence for all pathogens (Fig. 4), except for *M. mercenariae*-like that exhibited the highest prevalence in spring 2018 (23.3%, $n = 14/60$). Furthermore, the five individuals with the highest intensity of infection ($C_t \leqslant 30$) with *V. aestuarianus* were observed during summer 2018. The highest prevalence of coinfection with Haplosporidia and *Vibrio* spp. occurred in summer 2018 (21.7%, $n = 35/161$), while the highest coinfection prevalence with *M. tapetis* and *M. mercenariae*-like occurred in spring 2018 (11.7%, $n = 7/60$), with no occurrence in winter 2018/19. The higher pathogen incidence during spring and summer corresponded with higher seawater temperature and a peak in the presence of epiflora on the cockle shells (Table 8). The other two-pathogen coinfections with *Minchinia* species and *V. aestuarianus* showed no significant differences throughout the year [P(Chisq) > 0.05] since the total number of positive cases detected was too low [*M. tapetis* and *V. aestuarianus* ($n = 6$); *M. mercenariae* and *V. aestuarianus* ($n = 3$)]. There was only one case of three-pathogen coinfection with *M. tapetis*, *M. mercenariae*-like and *V. aestuarianus* in spring 2019 (0.6%, $n = 1/162$).

Histopathology

The histological screening was used as a validation method of the PCR and qPCR results. Haplosporidian infection was observed in histological sections of 67 cockles out of a subsample of 70

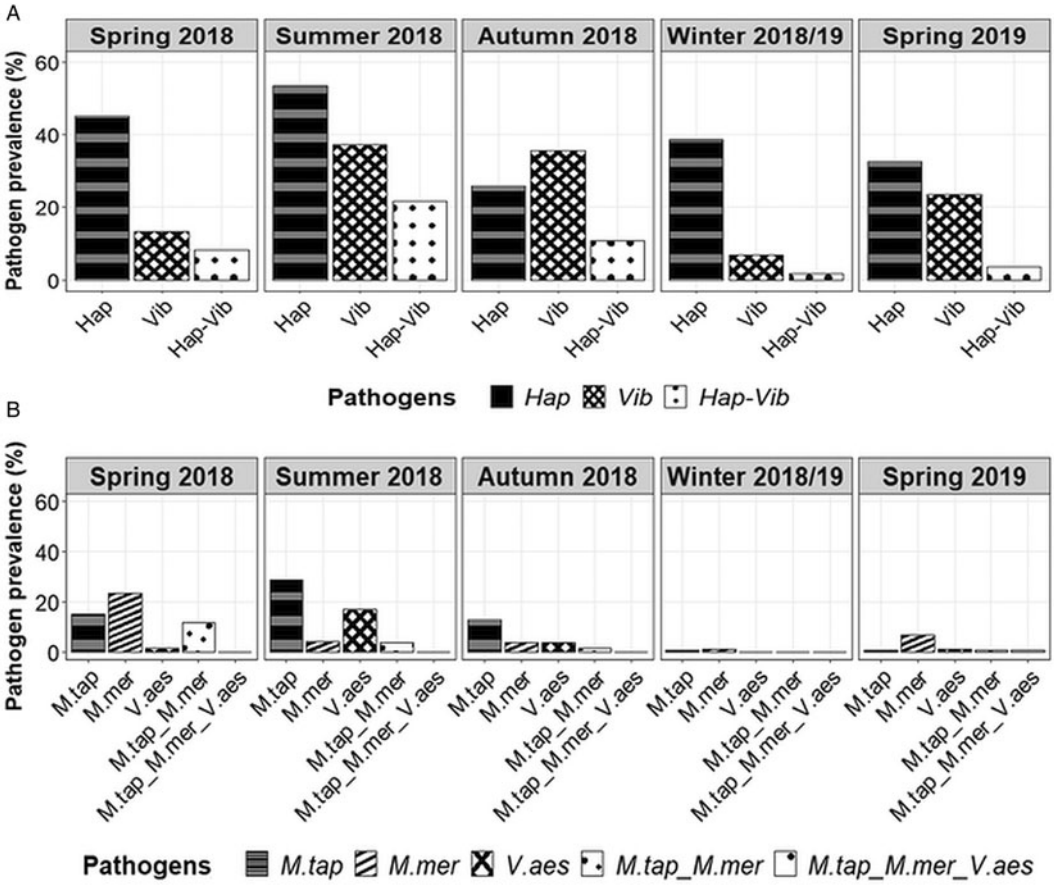

Fig. 4. (A) Prevalence (%) of Haplosporidia (Hap) and *Vibrio* (Vib) single infections and coinfection (Hap-Vib) by season. (B) Prevalence (%) of *Minchinia tapetis* (M.tap), *M. mercenariae*-like (M.mer) and *V. aestuarianus* (V.aes) single infections and coinfections (M.tap_M.mer and M.tap_M.mer_V.aes) by season.

samples (95.7% visual confirmation) that were positive for generic haplosporidian PCR. Mostly haplosporidians were observed as multinucleate plasmodia, multiple haplosporidia-like sporonts and developing spores similar to those described for *Minchinia* spp. (Ramilo *et al.*, 2018; Lynch *et al.*, 2020*b*; Fig. 5), and as uninucleate cells stage in lower numbers. Among the sample sites, the haplosporidia-like sporonts were predominantly visualized in the infected individuals from Cork Harbour in spring 2018, autumn 2018 and spring 2019; while the developing spores were observed mainly in the infected individuals from Dundalk Bay in summer 2018 and winter 2018/19. A higher prevalence of haplosporidians was recorded by PCR at both sites. The multinucleate plasmodia were visualized in cockles throughout the year at the different sample sites. The multinucleate plasmodia and haplosporidia-like sporonts were mainly observed throughout the connective tissue of the gills and digestive gland, while the developing spores were principally found in the mantle of infected animals.

Host pathology associated with *Vibrio* species included haemocyte accumulation in the connective tissues, as McCleary and Henshilwood (2015) described in *C. gigas* infected with *V. aestuarianus*. In this study, some of the sections examined ($n = 28$) showed haemocyte accumulation in the connective tissues of *C. edule* (Fig. 5). However, the presence of haemocyte infiltrations throughout the organs and tissues has also been previously associated with *Minchinia* infection (Longshaw and Malham, 2013). In this study, only in a minor number of screened samples ($n = 4$), where *Vibrio* and Haplosporidia were detected by PCR, haemocyte infiltrations were observed, all of them at Cork Harbour.

Although OsHV-1 μVar is not visible using histology, cytopathic effects can be observed in infected hosts, such as hypertrophied cells with a low nucleus-cytoplasm ratio and nuclear abnormalities (Prado-Alvarez *et al.*, 2016). Abnormal cell pathology associated with a viral infection was not observed in the histology ($n = 70$). Similarly, microsporidial spores may be visualized by microscopy in infected hosts (Garcia, 2002); however, it was not observed in the histological sections screened ($n = 70$).

Ten histological sections of samples that were negative by PCR were screened and no evident signal of infection was found in those, and tissues, organs and sinuses presented good integrity and a clear structure.

Discussion

The present study describes for the first time, to the best of our knowledge, simultaneous infections with haplosporidians and *Vibrio* spp. in wild and fished *C. edule* populations along the Irish coast. Coinfections with two and, especially, three pathogens, were rare in this study compared to single infected individuals. This finding may indicate that coinfected individuals succumb more easily to simultaneous infections and die. The lower number of coinfected individuals screened might indicate that the sampling was carried out on a much smaller cohort of survivors within the population. This survival bias is supported by previous studies on mortalities associated with coinfections of ostreid herpesvirus (OsHV-1 and variants) and *Vibrio* spp. in *C. gigas* in France (Petton *et al.*, 2015; Azéma *et al.*, 2016; Petton *et al.*, 2019), as well as with the co-occurrence of different protozoa and bacteria in the noble pen shell *Pinna nobilis* in the Mediterranean Sea (Carella *et al.*, 2020). Only three individuals presented a three-pathogen coinfection with *Minchinia* spp. and *Vibrio* spp., all located at Cork Harbour. Cork Harbour was the site with the oldest cockles, and it is well known that older

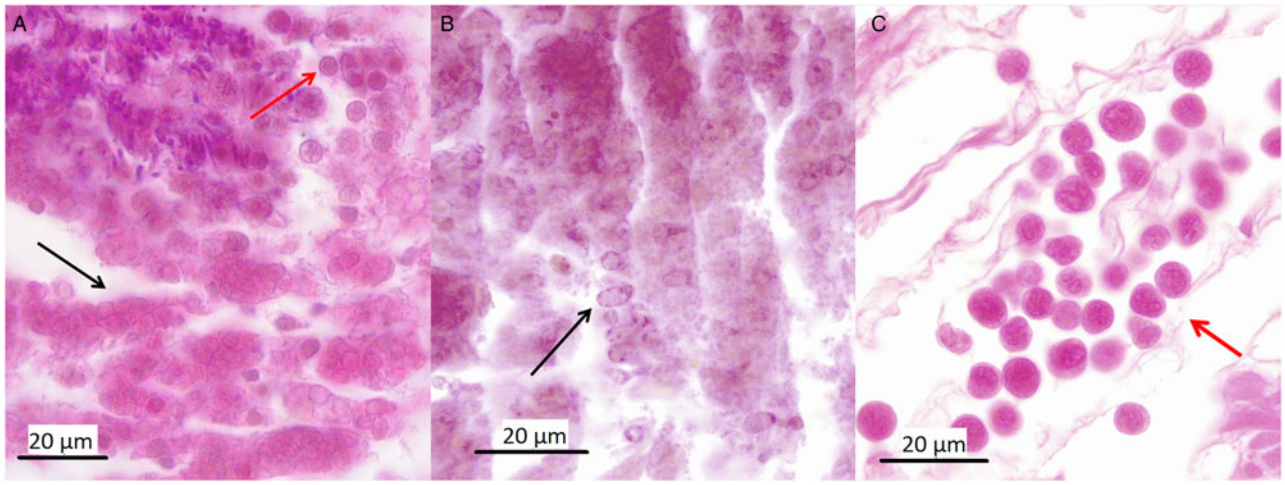

Fig. 5. (A) Multiple haplosporidia-like sporonts (black arrow) along with haemocytes (red arrow) in the connective tissue of *Cerastoderma edule*; (B) a large number of haplosporidian spores (black arrow) in the mantle tissue of *C. edule*; and (C) red arrow shows a haemocyte accumulation in the connective tissue of *C. edule*.

hosts have more time than younger hosts to accumulate disease-causing pathogens (Lafferty and Kuris, 2009; Breitburg *et al.*, 2015). Nevertheless, older bivalves have also more time available to develop immunotolerance and defence mechanisms compared to their younger cohorts (Renault and Novoa, 2004; Coen and Bishop, 2015). It may be the reason why cockles at Cork Harbour have not succumbed to the multiple infections.

Even though Haplosporidia and *Vibrio* were found simultaneously in double infected individuals, no significant biological interaction was statistically identified between those pathogen groups. Unlike other studies that have reported that multiple pathogens can interact synergistically, with the presence of one pathogen enhancing the abundance and/or virulence of the other (De Lorgeril *et al.*, 2018; Carella *et al.*, 2020). Concurrent infections by multiple pathogens species are more likely to occur in immune depressed individuals, which are more vulnerable to multiple opportunistic infections (De Lorgeril *et al.*, 2018; Carella *et al.*, 2020). These findings highlight that there can be variation amongst pathogen groups and the primary pathogen–opportunistic pathogen interplay.

It is well known that environmental and ecological factors potentially influence the mechanisms of disease transmission in marine systems, i.e. hydrodynamics, the biomass of infected animals, etc. (Petton *et al.*, 2015). Similarities in the host environment, behaviour or susceptibility can cause correlations in the risk of infection between two parasites (Vaumourin *et al.*, 2014). In this study, the proportion of double infected individuals with Haplosporidia and *Vibrio* was explained by risk factors (increased seawater temperature, reduced salinity and host condition) shared by the two pathogen groups, increasing the probability of infection by both infectious agents. Such confounding factors may influence host exposure and/or host susceptibility promoting simultaneous infections, even though involved pathogens do not interact biologically (Vaumourin *et al.*, 2015). These factors may be naturally occurring or be outcomes of coastal development (e.g. pollution) or climate change (e.g. warming, increased precipitation events and subsequent freshwater loadings into bays and estuaries), and may impact the expression of diseases directly or indirectly (Coen and Bishop, 2015). The predicted warming, increased precipitation and reduced salinity of coastal waters in temperate regions will provide new areas for the natural occurrence of pathogenic strains and may also impact the resilience of bivalve hosts (Rowley *et al.*, 2014; Lynch *et al.*, 2020*a*).

Consequently, spatial variability in the pathogen infection was detected driven by environmental and biological variables. The highest prevalence of coinfection with Haplosporidia and *Vibrio* spp. occurred in Dundalk Bay, which had a lower mean salinity throughout the year compared to the other sites. Gorrasi *et al.* (2020) observed that the abundance of *Vibrio* genus in water decreased along the salinity gradient within a marine saltern hypersaline environment. In general, marine vibrios are considered halotolerant, with no extreme halophiles reported within this genus (Gorrasi *et al.*, 2020). Previous studies have highlighted the intolerance of haplosporidians to low salinity in *C. edule* (Albuixech-Martí *et al.*, 2020), eastern oyster *Crassostrea virginica* (Ford and Haskin, 1982; Burreson and Ford, 2004) and European flat oyster *Ostrea edulis* (Flannery *et al.*, 2014). The high prevalence of haplosporidians at Dundalk Bay may be due to its commercial fishery activity. It is recognized that fishing and dredging may unbalance the interplay between infectious disease agents and their hosts in marine ecosystems (Rowley *et al.*, 2014; Coen and Bishop, 2015). Cranfield *et al.* (1999, 2005) hypothesized that extensive dredging in Foveaux Strait of New Zealand contributed to increased densities of Chilean oysters (*Ostrea chilensis*) and decreased densities of other filter-feeding organisms, that may have previously reduced the dispersal stages of the haplosporidian *Bonamia exitiosa*, enhancing disease transmission. The high cockle density found at Dundalk Bay may have facilitated the infection among the cockle population, due to the proximity of infected to non-infected cockles. The cultivated and harvested molluscs tend to have a much greater incidence of disease than wild molluscs, which may be because they are placed at high densities which enhance disease transmission and cause stress (competition for resources, space, etc.) among individuals (Ford, 2002).

Dungarvan Harbour also displayed a high prevalence of coinfection with Haplosporidia and *Vibrio* spp. compared to the other sites. Cockles from Dungarvan Harbour were the smallest and the youngest ones. Although it is accepted that older hosts have more time to accumulate disease-causing pathogens (Lafferty and Kuris, 2009; Breitburg *et al.*, 2015), the susceptibility of small bivalves to viral and bacterial infections is generally greater than for larger adults (Renault and Novoa, 2004; Coen and Bishop, 2015). Moreover, the proximity of an extensive *C. gigas* culture site at Dungarvan Harbour, where *Vibrio* spp. are endemic (pers. comm. with Marine Institute Ireland), can have promoted the presence of *Vibrio* in the environment. Particularly, *V. aestuarianus* and *V. splendidus*, which have been identified in this study, have been described as increasingly harmful pathogens of *C. gigas* aquaculture (Le Roux *et al.*, 2002; Garnier *et al.*, 2007; McCleary and Henshilwood, 2015). Caballes *et al.* (2012)

demonstrated interspecific transmission of *Vibrio rotiferianus* between co-occurring echinoderm species.

Even though the natural host for OsHV-1 are *C. gigas*, this virus can behave opportunistically and infect other cohabiting bivalve species (Bookelaar *et al.*, 2020). It has been previously reported in *C. edule*, in Ireland (Bookelaar *et al.*, 2020) and in the Sydney cockle, *Anadara trapezia*, in Australia (Evans *et al.*, 2017). Nevertheless, no presence of OsHV-1 μVar was detected in the study, despite the extensive farming of *C. gigas* at Dungarvan Harbour, which has a history of OsHV-1-associated mortality events (Lynch *et al.*, 2012; Prado-Alvarez *et al.*, 2016). During summer 2018, the prevalence of OsHV-1 μVar in *C. gigas* at Dungarvan Harbour was confirmed by Kett *et al.* (unpublished data) to be 48.4% ($n = 92/190$), with low mortality rates (max. of 6% at the high shore in July). Moreover, Bookelaar *et al.* (2020) observed OsHV-1μVar-infected *C. edule* (14.4%; $n = 36/250$) and infected *C. gigas* (range of 0–27% per month; $n = 270$) at Dungarvan Harbour from April 2015 to August 2015, with no recording of mortalities. Thus, the lack of OsHV-1 μVar detection in *C. edule* at Dungarvan Harbour in the current study may be because the virus remained within the oysters and was not shed from dying or dead oysters into the environment, as the high prevalence and low mortality recorded by Kett *et al.* (unpublished data) suggested. The smaller sample size ($n = 81$), compared to the above studies, taken during spring and summer 2018, when the OsHV-1 μVar can have a major emergence due to the higher temperatures (Renault *et al.*, 2014), may have also influenced the results.

Seasonal variability was also observed in this study, with the highest prevalence of coinfection with Haplosporidia and *Vibrio* spp. occurring in summer 2018, which coincided with the highest intensity of infection with *V. aestuarianus.* Additionally, lower occurrences of the haplosporidian and *Vibrio* species quantified were detected in the cooler spring 2019 compared to spring 2018 in this study. Burreson and Ford (2004) tested that low-temperature conditions caused a dramatic reduction in the prevalence of *Haplosporidium nelsoni* in *C. virginica*. Conversely, a higher prevalence of *Bonamia ostreae* in European flat oyster *O. edulis* was associated with cooler spring temperatures over a 30-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. It is also well-known that bacteria belonging to the genus *Vibrio* are strongly thermodependent and their occurrence is expected to rise with increasing temperature conditions (Garnier *et al.*, 2007; Cotter *et al.*, 2010; Vezzulli *et al.*, 2010). Most of the reported *Vibrio*-associated mass mortalities in marine invertebrates have occurred during the summer when seawater temperatures were higher than 18°C (Le Roux *et al.*, 2002; Paillard *et al.*, 2004; Garnier *et al.*, 2007; Vezzulli *et al.*, 2010). Therefore, high temperatures in summer might have triggered a major pathogen propagation and, consequently, to a higher prevalence of two-pathogen coinfection. While lower temperatures in winter, with the lowest prevalence of two-pathogen coinfection, would have kept the pathogens at a sub-lethal level in the hosts reducing the transmission between cockles. It has been observed that *Vibrio* spp. may enter into a dormant state when the environmental conditions become unfavourable (i.e. suboptimal or reduced temperature, elevated salinity, low nutrient concentration and extreme pH) (Lin and Schwarz, 2003; Nowakowska and Oliver, 2013; Neogi *et al.*, 2018). The low number of coinfected individuals in winter might also suggest that most *C. edule* had succumbed to infection in autumn after the peak in summer.

It was also observed that with the higher temperatures in summer and spring, the presence of epiflora on the cockle shells was more frequent, which may have consequences on cockle susceptibility to infectious agents. As seen in shellfish aquaculture, the presence of biofouling on the stock can have a direct impact, causing physical damage, biological competition, mechanical interference and environmental modification, while infrastructure is also impacted (Fitridge *et al.*, 2012). Microalgae have been pointed as a repetitive and substantial source of bacterial inoculation into the bivalve larval culture systems (Salvesen *et al.*, 2000). Microalgae may promote and/or inhibit bacterial growth by the production of organic exudates and toxic metabolites (Salvesen *et al.*, 2000). For instance, *Vibrio* species such as *V. splendidus* or *V. parahaemolyticus* have been found to be associated with microalgae (Kumazawa *et al.*, 1991; Rehnstam-Holm *et al.*, 2010; Notaro *et al.*, 2021). Therefore, the presence of epiflora on the shells might have also exposed the cockles to pathogens, in particular *Vibrio* species.

Both species of *Minchinia*, *M. tapetis* and *M. mercenariae*-like, were found to occur together significantly more often than expected by random chance, indicating a positive two-way association between both species. However, previous studies examining pairwise coinfections indicate that antagonistic interactions between similar types of parasites may be common (Dobson, 1985; Johnson and Hoverman, 2012). A possible explanation of the established synergetic interaction is that, despite both *Minchinia* species being closely related, they exhibit differences in their pathogenic effect and use of resources within the host. Carballal *et al.* (2020) observed that the distribution of infection with *M. tapetis* and *M. mercenariae*-like haplosporidian in cockles *C. edule* in Galicia was different throughout the cockle tissues. *M. mercenariae*-like haplosporidian was observed in the connective tissue of the digestive gland, gills and gonad, while *M. tapetis* was only observed in the digestive gland (Carballal *et al.*, 2020). Further research is needed to reveal the underlying mechanism of this synergetic association in *C. edule* and the role that host exposure and host habitat play in it.

In brief, coinfected individuals may more easily succumb to pathogen load. The proportion of double infected individuals with haplosporidian and *Vibrio* was driven by environmental and biological factors, triggering a spatial and seasonal variability in the distribution of coinfection. The higher temperature in warmer months and the presence of epiflora, which can be associated with pathogens, on the cockle shells may have promoted the risk of coinfection, along with low salinity and the host condition in some of the sample sites. The close proximity to other infected host species may have also influenced the coinfection distribution in *C. edule*. Similarly, fished *C. edule* populations with high cockle densities might have been more exposed to the infection. Therefore, those factors may conform the ideal scenario for simultaneous infections, although the pathogen groups showed no biological interaction between them. Nevertheless, a positive two-way association between both *Minchinia* species was observed, which is suggested to be due to their different pathogenic effect and use of resources within the host. Our findings shed light on the complex interactions between multiple aetiological agents associated with host diseases in the frame of a natural system. This study highlights the consequences of the coinfections are not simply the sum of the effects caused by each infectious agent, but the result of a complex combination of direct and indirect pathogens effects, the host ecology and the environmental context.

These results are framed into the current scenario of climate change, which involves oceans warming on average by 0.6°C in the next 100 years (Wiltshire *et al.*, 2008), with European seas especially affected (Philippart *et al.*, 2011). The sea surface temperature around the UK and Ireland has increased at six times the rate of the global average, particularly jeopardizing species that have small thermal tolerance windows (Frost *et al.*, 2012). As a result, previously appropriate habitats for cockles may no longer be suitable due to changes in thermal regime, oxygen levels or altered rainfall

patterns (Morgan *et al.*, 2013; Coen and Bishop, 2015). Climate change may be also expected to have a large effect on disease occurrence, especially in host species showing strong relationships between temperature/salinity and diseases, as seen for the cockle populations studied, and for many other molluscan host–parasite systems (Allam and Raftos, 2015; Coen and Bishop, 2015; Lynch *et al.*, 2020*a*). The physiological stress may also cause the host to become immune-compromised and unable to suppress an infection (Suttle, 2007). Therefore, climate change could be the driver of the spreading and range expansions of infectious agents, such as bacteria, haplosporidians and viruses, in the marine ecosystem and may lead to more frequent pathogen outbreaks (Burreson and Ford, 2004; Suttle, 2007; Rowley *et al.*, 2014; Coen and Bishop, 2015). As global climate change and anthropogenic impacts continue to modify the distribution and abundance of hosts and parasites and the ways in which they interact, integral studies like ours are important since concepts gained might be applied to other associations and to a changing environment.

A final important point to emphasize is that the relative potential of coinfections to cause the emergence of zoonoses in wild animals remains to be fully assessed (Hoarau *et al.*, 2020). Given that two-thirds of emerging infectious diseases are zoonoses, with nearly 70% originating from wildlife (Hoarau *et al.*, 2020), better knowledge of the interactions of infectious agents in wild reservoirs, such as cockles, will provide key insight for the understanding and management of spillover processes. Although challenging, findings from this study highlight the necessity to integrate data pertaining to host ecology, pathogen diversity, biogeography and seasonality of the disease and the environmental influence. This approach should be consistently applied to provide a more detailed picture of coinfection dynamics in individual hosts and communities over time.

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Connectivity dynamics in Irish mudflats between microorganisms including *Vibrio* spp., common cockles *Cerastoderma edule*, and shorebirds

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Shellfish, including the key species the common cockle *Cerastoderma edule*, living and feeding in waters contaminated by infectious agents can accumulate them within their tissues. It is unknown if microbial pathogens and microparasites can subsequently be transmitted via concomitant predation to their consumers, including shorebirds. The objective of this study was to assess if pathogens associated with *C. edule* could be detected seasonally in the faeces of shorebirds that feed on *C. edule* and in the physical environment (sediment) in which *C. edule* reside, along the Irish and Celtic Seas. Two potentially pathogenic global groups, *Vibrio* and Haplosporidia, were detected in *C. edule*. Although Haplosporidia were not detected in the bird faeces nor in the sediment, identical strains of *Vibrio splendidus* were detected in *C. edule* and bird faecal samples at sites where the oystercatcher *Haematopus ostralegus* and other waders were observed to be feeding on cockles. *Vibrio* spp. prevalence was seasonal and increased in *C. edule* and bird faecal samples during the warmer months, possibly due to higher seawater temperatures that promote the replication of this bacteria. The sediment samples showed an overall higher prevalence of *Vibrio* spp. than the bird faecal and *C. edule* samples, and its detection remained consistently high through the sites and throughout the seasons, which further supports the role of the sediment as a *Vibrio* reservoir. Our findings shed light on the fact that not all pathogen groups are transmitted from prey to predator via feeding but bacteria such as *V. splendidus* can be. As most of the wading birds observed in this study are migratory, the results also indicate the potential for this bacterium to be dispersed over greater geographic distances, which will have consequences for areas where it may be introduced.

Mudflats are coastal wetlands that form in sheltered intertidal areas such as bays and estuaries, where sediments have been deposited by tides or rivers. Mudflats support a large population of wildlife and are a key habitat for many migratory shorebirds, as well as for certain species of molluscs, crabs, and fish[1]. The common cockle *Cerastoderma edule* is widely distributed in the Atlantic and one of the main non-cultured bivalve species harvested in western European bays and estuaries, where population densities of 10,000 per m^2 have been recorded[2], making them a useful model species for this study. Furthermore, common cockles play a key role as an ecosystem engineer, controlling or influencing processes such as bioturbation and water filtration, increasing the productivity of sedimentary habitats, which underpin marine food webs and biodiversity[3–5]. In the marine food chain, *C. edule* is an important food source and are a link between primary producers (phytoplankton, phytobenthos) and consumers such as crabs, shrimps, fish and birds[4]. Cockles, inhabiting the intertidal, are easily available to a variety of predators since they are found only a few millimetres beneath the sediment surface, they may even emerge at the surface when abundance is high, because of sediment erosion, or when they are stressed, such as by oxygen deficiency or trematode infection[6]. One of the main consumers feeding on up to 300 cockles by day is marine bird populations[3], whose feeding ecology may shape their diverse and multiple endoparasitic fauna[7], responsible

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for significant effects on marine birds[8]. Trophically transmitted parasites and pathogens are central elements in most aquatic food webs, given that foraging behaviour and diet facilitate the transmission of a variety of pathogens and endoparasites through marine invertebrate and vertebrate species[9,10]. Concomitant predation is the most common way for parasites and pathogens to become prey, which occurs when an infected host is eaten by a predator. Because of the high productivity of aetiological agents, their unique nutritional composition and their pathogenicity in hosts, their consumption affects food web structure, energy transfer and disease dispersals[11].

Although parasitism contributes significantly to the biodiversity of mudflat ecosystems, the composition of parasites in the food web of the mudflat ecosystems has been understudied[1]. Cockles inhabiting those habitats can accumulate agents that are potentially pathogenic[12] and act as a reservoir for subsequent infection of other species[5]. Cockles are hosts to a diverse range of macroparasites[13], but may be also infected by a range of pathogens including protists, bacteria and viruses[14]. In turn, Newman et al.[15] confirmed that infectious diseases are an important cause of mortality events and individual bird deaths, especially among coastal and freshwater aquatic birds, pointing out bacterial infections as a frequent cause of mass mortality events.

A wide range of bacterial infections occurs in free-ranging birds[8], with many copiotrophic bacteria, such as *Vibrio* spp., widely distributed in estuarine and coastal ecosystems[16,17]. A large number of *Vibrio* species are associated with marine organisms like fish, molluscs and crustaceans, in commensal or pathogenic relations[18]. Some species such as *Vibrio aestuarianus*, *Vibrio splendidus* or *Vibrio tapetis* have been associated with mortalities of different molluscan species, seriously affecting their culture and causing high losses in hatcheries as well as in natural beds[19–24]. For other species, ecological importance has been demonstrated, such as *Vibrio crassostreae, Vibrio breoganii, Vibrio celticus*, which form part of the molluscan microbiota[18], or *V. mediterranei*, found in the microbiota of marine fish[25]. In addition, *Vibrio* bacteria can persist in high abundance and/or can be found during cold and unfavourable environmental conditions in the sediment compartment[26,27]. Azandégbé et al.[28] reported the occurrence of *V. aestuarianus* in sediment at two *C. gigas* farms in France and suggested that this bacterium might subsist during the cold seasons in the sediment and rise again under favourable conditions.

The haplosporidian protists are also widespread and have been associated with some of the most serious epizootic mortalities of commercially important bivalves, including cockle population crashes in The Netherlands and UK[14,29–32]. A number of studies have reported the detection of *Minchinia*[14,30,33–35] and *Haplosporidium* in cockles[30,36], and *Urosporidium* parasitizing trematodes or turbellaria infecting cockles[37]. Cockles are a known food source for shorebirds; however, to the best of our knowledge, Haplosporidia have not been previously associated with birds.

Viral diseases play an increasingly important role in the health of aquatic birds, as has been suggested in previous studies[38–40]. Particularly, herpesviruses have been previously associated with respiratory and enteric disease and mortality among seabirds and waterfowl[41–43]. In turn, even though the natural host for ostreid herpesvirus type-1 (OsHV-1) are oysters, Bookelaar et al.[44] recently determined the potential for *C. edule* to become infected with and to act as an alternate host of OsHV-1 µVar. The presence of herpesviruses in the sediment has been also described. Honjo et al.[45] reported for the first time Cyprinid herpesvirus 3 (CyHV-3) DNA in sediments of natural lakes and ponds suggesting that sediment could act as a reservoir for CyHV-3 in natural freshwater environments. Subsequently, Evans et al.[46] detected OsHV-1 in the mangrove sediments from an estuary in Australia.

Therefore, the environmental reservoirs along with the hosts may largely influence the ecology of those pathogen groups by favouring their survival and dispersion in the environment and also serving as a vector for their associated disease. Free-living birds might be involved in the carriage of microbial pathogens not only as biological carriers (the pathogen multiplies in the avian body) but also as mechanical carriers (the pathogen does not multiply in or on the bird)[41]. The pathogen can be located on the surface of the bird's body or pass through the digestive tract, being viable when excreted[41]. Likewise, birds are hosts for many parasites that sometimes serve as vectors of infectious agents[41]. Birds, including waterfowl, can be hyperparasitized by microsporidians that infect their parasites[47,48]. Hyperparasitism by microsporidia might influence trophic relationships between invertebrate hosts such as *C. edule* and vertebrate hosts such as birds, releasing the secondary host from metacercarial infections[49]. Therefore, birds may play an important role as a reservoir of infection and/or incidental carrier influencing pathogen transmission, dispersal, and disease dissemination. The potential for transport and dissemination of certain pathogenic microorganisms by free-living birds is of concern and is the subject of increased vigilance. For instance, the roles of wild birds in the spread of avian flu and tick-borne viruses are well recognised[50,51].

Parasite-inclusive food web studies have so far mainly considered macroparasites; however, the large diversity of microparasites and pathogens in mudflat ecosystems remains to be fully explored[1]. Even though it is well-known that infectious agents may incur costs for their avian hosts at an individual level[8], the trophic transmission of microbial and parasitic infections in aquatic birds and their influence on disease dynamics and dissemination are difficult to establish. Further complications arise from the fact that some host groups such as birds are often transient components of the local food web that can carry parasites and pathogens acquired elsewhere[1]. Polymerase chain (PCR)-based techniques for detecting prey remains in the gut, faeces and regurgitates of predators can be applied to study complex trophic interactions in the field; however, their application is still a challenge. The lack of sensitivity, short post-ingestion detection periods and cross-amplification problems are the major difficulties for detecting degraded, semi-digested DNA[52]. Nevertheless, following stringent contamination control strategies when collecting samples, prompt preservation of those samples and assay optimization can prevent these problems[53–56].

The presence of the *Vibrio* genus in *C. edule* has been confirmed on the eastern and southern Irish coasts[57]. Likewise, there have been previous records of infected *C. edule* populations with haplosporidian protists such as the genus *Minchinia*[34,35], or with ostreid herpesvirus type-1 (OsHV-1)[44], as well as records of microsporidian species parasitizing digeneans that infect cockle populations in those areas[13,49]. The objective of this study was to assess if pathogens associated with *C. edule* (*Vibrio* spp., Haplosporidia spp., ostreid herpesvirus-1 microVar,

Microsporidia spp.) could be detected seasonally in the sediment and the faeces of shorebird species at sample sites along the Irish and Celtic Seas. This study used PCR-based techniques adopting strict contamination control strategies and optimisation of the storage, extraction, and purification of the DNA from bird faecal droppings, sediment, and cockles. Shorebird species present were recorded while foraging, with particular attention to cockle consumption. The observational data from shorebirds was assessed in relation to *C. edule* DNA presence and pathogen detection in faecal samples. The spatial and seasonal influence in the detection of the infectious agents in cockles, shorebird faecal samples as well as sediment was also evaluated. Findings will provide an insight into the pathogen community present in *C. edule*, shorebird populations and sediment, establishing the potential of the different pathogen groups to persist in these three natural compartments and be trophically transmitted from *C. edule* to shorebirds. This study highlights the role of the shorebirds as carriers of infectious agents as well as the role of the sediment in the pathogen persistence.

Results

Bird species and observational studies. A detailed list of the bird species identified as well as the behaviour observed in the recording is provided in Supplementary Table S1, likewise, their diet and status are described in Supplementary Table S2.

Overall, the bird presence on the sampled cockle beds, in the intertidal and subtidal areas, was constant at the sample sites and throughout the year (Supplementary Table S1). The only exception was in spring 2019 in Annagassan, where there was a clear disturbance in the shorebird community due to a flying drone in the area. A variety of waders (Supplementary Table S1)—oystercatchers *Haematopus ostralegus*, curlews *Numenius arquata*, bar-tailed godwits *Limosa lapponica,* black-tailed godwits *Limosa limosa,* common redshanks *Tringa totanus*, red knot *Calidris canutus* and dunlins *Calidris alpina*—that feed on bivalves, including cockles, were identified in the recordings along with several species of seagulls—*Larus canus, Chroicocephalus ridibundus, Larus marinus, Larus melanocephalus*—and hooded crows (*Corvus cornix*), both groups with a generalist diet but that can feed on bivalves in coastal areas (Supplementary Table S2). Most waders observed were actively foraging and feeding (Table 1; Supplementary Table S1). Some recording was done of birds, especially oystercatchers, digging out shellfish from the sediment and opening them with the beak. Gulls, in turn, were usually loafing, preening, and passively foraging and feeding (Table 1; Supplementary Table S1).

The sample sites with lower bird occurrence were Ringaskiddy and Youghal, where a few scattered bird individuals were recorded (mainly *H. ostralegus*, *N. arquata* and *L. canus*), except for a flock of *C. canutus* ($10 \leq 20$ individuals) in summer 2018 at Youghal (Table 1; Supplementary Table S1). Bird flocks (> 10 individuals) were common in Cuskinny, Dungarvan, and Dundalk (Annagassan and Cooley) (Table 1; Supplementary Table S1). Large gull flocks (> 20 individuals) were recorded at Cooley and, especially, at Cuskinny and Dungarvan (Table 1; Supplementary Table S1). In turn, wader flocks ($10 \leq 30$ individuals), including *H. ostralegus*, *L. limosa*, *T. totanus* and *C. alpina*, were more frequently recorded in Dundalk sites (Annagassan and Cooley) (Table 1; Supplementary Table S1). Gulls were recorded over the year, except for the winter season in Annagassan and Cooley, while waders seemed to be more frequently observed during autumn and winter (Supplementary Table S1), which would correlate with their status as winter visitors to Ireland (Supplementary Table S2).

Molecular cockle species identification. All the screened cockles were identified as *Cerastoderma edule*, with no presence of *Cerastoderma glaucum* or hybrids.

Among the faecal and sediment samples taken, 61.8% (n = 126/204) of the faecal samples and 63.2% (n = 129/204) of the sediment samples showed good DNA quantity (≥ 1 ng/µl) and high-quality DNA (A260/A280 = 1 ≤ 1.99), while 17.6% (n = 36/204) of the faecal samples and 18.1% (n = 37/204) of the sediment samples showed good DNA quantity (≥ 1 ng/µl) and presence of RNA along with DNA (A260/A280 = 2 ≤ 4).

In the seabird faecal samples, 22.5% (n = 46/204) contained *C. edule* DNA (Fig. 1), with 63.0% (n = 29/46) of those samples displaying high quantities of DNA (≥ 1 ng/µl) and high-quality DNA (A260/A280 = 1 ≤ 1.99). In the sediment samples, 33.8% (n = 69/204) contained *C. edule* DNA (Fig. 1), with 56.5% (n = 39/69) of those samples displaying good quantities of DNA (≥ 1 ng/µl) of high-quality (A260/A280 = 1 ≤ 1.99). There was no presence of *C. glaucum* DNA or hybrids in any seabird faecal or sediment samples.

The highest percentage of *C. edule* DNA detected in faecal samples occurred at Dundalk Bay – Annagassan (30.6%; n = 11/36) and Cooley (30.0%; n = 12/40) –, where the cockle abundance found was high compared to the other sites and where oystercatchers *H. ostralegus*, curlews *N. arquata*, black-tailed godwits *L. limosa*, common redshanks *T. totanus* and dunlins *C. alpina* were observed foraging (Fig. 2). Cuskinny, where gulls were the dominant species recorded and the cockle abundance observed in that area was lower than other sites, was the site with the lowest level of *C. edule* DNA found in faeces (12.5%; n = 4/32) (Fig. 2). Youghal, with a low cockle and bird abundances observed, also showed a low detection of *C. edule* DNA in faeces (14.7%; n = 5/34) (Fig. 2). The lowest *C. edule* DNA detection in the sediment samples was in Ringaskiddy (20.6%; n = 7/34), where a low abundance of cockles and empty cockle shells were found at every sampling, while the highest detection was in the commercial fishery of Dundalk—Annagassan (36.1%; n = 13/36) and Cooley (60.0%; n = 24/40) -, with higher cockle abundances observed (Fig. 2).

Seasonal variability in the detection of *C edule* DNA in faecal and sediment samples was observed, with the highest detection of *C edule* DNA during spring 2018 (faeces: 100%, n = 6/6; sediment: 83.3%, n = 5/6) and summer 2018 (faeces: 73.9%, n = 17/23; sediment: 78.3%, n = 18/23), when more screened cockles were found dead or in poor condition (full of sand and bad smell); while the lowest detection was in winter 2018/19 (faeces: 6.7%, n = 4/60; sediment: 20.0%; n = 12/60) (Fig. 3). Flocks of *H. ostralegus, C. alpina* and *C. canutus* ($10 \leq 20$ individuals) were observed during summer, while *H. ostralegus*, *T. tetanus*, and *L. limosa* flocks ($10 \leq 30$ individuals)

Sites	Sampling dates	Tidal zonation	Bird species	Number of individuals	Behaviour*
Ringaskiddy	Spring 2018	Intertidal	Common gulls (*Larus canus*)	5	O
	Autumn 2018	Intertidal	Oystercatchers (*Haematopus ostralegus*)	3	F
	Winter 2018/19	Intertidal	Oystercatchers (*Haematopus ostralegus*)	5	F
	Spring 2019	Intertidal	Common gulls (*Larus canus*)	2	O
			Great black-backed gulls (*Larus marinus*)	2	
Cuskinny	Spring 2018	Intertidal	Hooded crows (*Corvus cornix*)	Flock (~ 10)	F/O
	Autumn 2018	Intertidal	Black-headed gull (*Chroicocephalus ridibundus*)	Flock (40–50)	F/O
	Winter 2018/19	Intertidal	Common gulls (*Larus canus*)	Flock (20–30)	F/O
			Hooded crows (*Corvus cornix*)	Flock (10–20)	
	Spring 2019	Intertidal	Black-headed gull (*Chroicocephalus ridibundus*)	Flock (30–50)	F/O
Youghal	Summer 2018	Intertidal	Red knot (*Calidris canutus*)	Flocks (10–20)	F
	Autumn 2018	Intertidal / subtidal	Common gulls (*Larus canus*)	4	O
	Winter 2018/19	Intertidal / subtidal	Oystercatchers (*Haematopus ostralegus*)	4	F
	Spring 2019	Intertidal	Oystercatchers (*Haematopus ostralegus*)	3	F
Dungarvan	Summer 2018	Intertidal	Oystercatchers (*Haematopus ostralegus*)	4	F
	Autumn 2018	Intertidal	Black-headed gull (*Chroicocephalus ridibundus*)	Flock (20–30)	F/O
	Winter 2018/19	Intertidal	Common gulls (*Larus canus*)	Flock (20–30)	O
			Great black-backed gulls (*Larus marinus*)	Flock (20–30)	
	Spring 2019	Intertidal	Oystercatchers (*Haematopus ostralegus*)	Flocks (~ 10)	F
Annagassan	Summer 2018	Intertidal	Dunlins (*Calidris alpina*)	Flock (10–20)	F
	Autumn 2018	Intertidal	Oystercatchers (*Haematopus ostralegus*)	Flocks (~ 10)	F
			Common redshank (*Tringa totanus*)	Flocks (~ 10)	
	Winter 2018/19	Intertidal	Oystercatchers (*Haematopus ostralegus*)	Flocks (10–20)	F
			Black-tailed godwits (*Limosa limosa*)	Flocks (10–20)	
	Spring 2019	Supratidal	Raven (*Corvus corax*)	1	F
Cooley	Summer 2018	Intertidal	Oystercatchers (*Haematopus ostralegus*)	Flocks (~ 10)	F
	Autumn 2018	Intertidal	Black-tailed godwits (*Limosa limosa*)	Flock (20–30)	F
	Winter 2018/19	Intertidal	Oystercatchers (*Haematopus ostralegus*)	Flocks (~ 10)	F
	Spring 2019	Intertidal / subtidal	Black-tailed godwits (*Limosa limosa*)	Flock (20–30)	F
			Common gulls (*Larus canus*)	Mixed flock (> 50)	F/O
			Mediterranean gulls (*Larus melanocephalus*)		
			Sandwich terns (*Sterna sandvicensis*)		

Table 1. The most abundant shorebird species, number, location and behaviour observed at each sample site by season, compiling the main flocks observed in the field. (*) Following the descriptions in Lewis and Tierney[58], shorebird behaviour was classified in two categories: (F) foraging/feeding: the active or passive search of food and feeding; and (O) other: all other behaviours that do not assign to foraging, including roosting, standing, preening, loafing, swimming and others.

along with large flocks of gulls (20 ≤ 50 individuals) and hooded crows (10 ≤ 20 individuals) were observed in autumn and winter (Fig. 3).

Pathogen screening. During the study two pathogen groups were detected in *C. edule* samples: 37.7% (n = 277/735) tested positive for Haplosporidia, while 25.3% (n = 186/735) tested positive for *Vibrio* spp. (Fig. 1; Table 2). Of the 277 cockles positive for Haplosporidia, 29.6% (n = 82/277) could be distinguished as being infected with *Minchinia tapetis* and 14.8% (n = 41/277) were infected with *Minchinia mercenariae*-like (Table 2). However, other haplosporidian species not targeted by this study could also be present in the *C. edule* samples

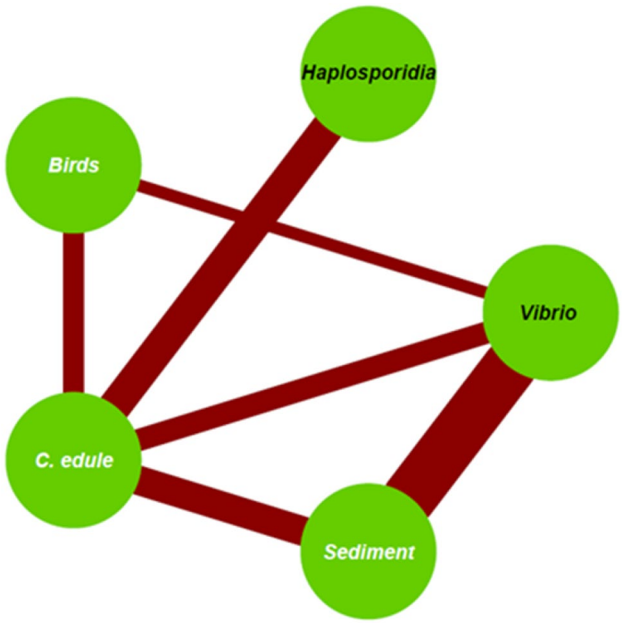

Figure 1. Network association plot displaying the links between the different compartments studied. The black text refers to pathogens and the white text relates to hosts/reservoirs. A solid line represents target DNA presence and line thickness indicates the prevalence (%) of infected *C. edule* and the percentage of positive faecal and sediment samples.

that were positive in the generic haplosporidian PCR. Of the 186 cockles positive for *Vibrio*, 19.9% (n = 37/186) were determined to be infected with *Vibrio aestuarianus* (Table 2), with highly infected individuals defined as those individuals that showed a $C_t < 30$, since the *V. aestuarianus* positive controls (diluted purified DNA) had a $C_t = 30$. Based on that, 13.5% (n = 5/37) of the individuals determined to be positive for *V. aestuarianus* were classified as heavily infected individuals, while 86.5% (n = 32/37) were classified as having light/low infections. Dungarvan Harbour was the site with the highest presence of *V. aestuarianus* and three out of the five heavily infected individuals were found in that site during summer 2018. Sequencing was conducted with a subsample of the other 80.1% (n = 149/186) of samples that were not positive for *V. aestuarianus*, results listed below (Table 3). No ostreid herpesvirus-1 microVar nor Microsporidia spp. were detected in any of the *C. edule* screened during the study (Table 2).

Of the various pathogen groups screened for, only vibrios were found in both bird faecal samples and sediment, apart from in *C. edule* samples (Fig. 1). *Vibrio* spp. DNA was detected in 13.7% of the seabird faeces (n = 28/204) and 62.7% (n = 128/204) of the sediment samples (Table 2). 64.3% (n = 18/28) of those faecal samples and 66.4% (n = 85/128) of those sediment samples displayed good quantities of DNA (≥ 1 ng/µl) and high-quality DNA (A260/A280 = 1 ≤ 1.99). The PCR positive samples for generic *Vibrio* in the three screened sample types were defined as *Vibrio* spp. for analysis purposes. However, specific screening of *V. aestuarianus* was conducted by qPCR and sequencing done with subsamples of the *C. edule*, faecal and sediment samples revealed other *Vibrio* species present during the study but not quantified.

V. aestuarianus was absent in the faecal samples, and it was only detected in two of the sediment samples (1.6%, n = 2/204), in spring 2018 at Cuskinny and in summer 2018 at Dungarvan (Table 2), and both were classified as having low bacteria loading. The strain of *V. aestuarianus* detected in cockles and sediment was confirmed to be the same (MK307696.1, strain 15_075_1T2), which has been recently confirmed to be pathogenic to the common cockles *C. edule*[59]. Other species of *Vibrio*, known as important aetiological agents of diseases affecting all life stages of shellfish[18,21], were also identified: *Vibrio splendidus* and *Vibrio kanaloae*. It was confirmed that three strains of *V. splendidus* identified by direct Sanger sequencing were the same in the three sample types (Table 3). The sequences obtained from the three samples types, with a query length between 87–155 bp, showed 60–91% query coverage and 92.6–98.7% percent identity to *V. splendidus* strains deposited by Gao (2020) (MT445179.1, strain FS1; MT445177.1, strain CT1) and by Landreau et al. (2020) (MT345091.1, strain D0-B01). Some of these sequences obtained from *C. edule* and bird faecal samples came from the same sampling site, Dungarvan (Table 3). However, the identified sequences from the sediment are from Ringaskiddy. Likewise, the *V. kanaloae* strain CAIM 485 was found by sequencing in both cockle and sediment samples (Table 3). The cockle samples, with a query length between 82–179 bp, showed 78–83% query coverage and 92.6–98.6% percent identity to *V. kanaloae* deposited by Sun (2020) (MT759943.1); while the sequence from the sediment sample, with a query length of 222 bp, showed 30% query coverage and 94.4% percent identity to *V. kanaloae* deposited by Sun (2020) (MT759943.1). Nevertheless, it was not considered a robust outcome due to the low query cover (30%) in the sequenced sediment sample. For two of the sequenced cockle samples, it was not possible to generate sequences that were long enough to identify species.

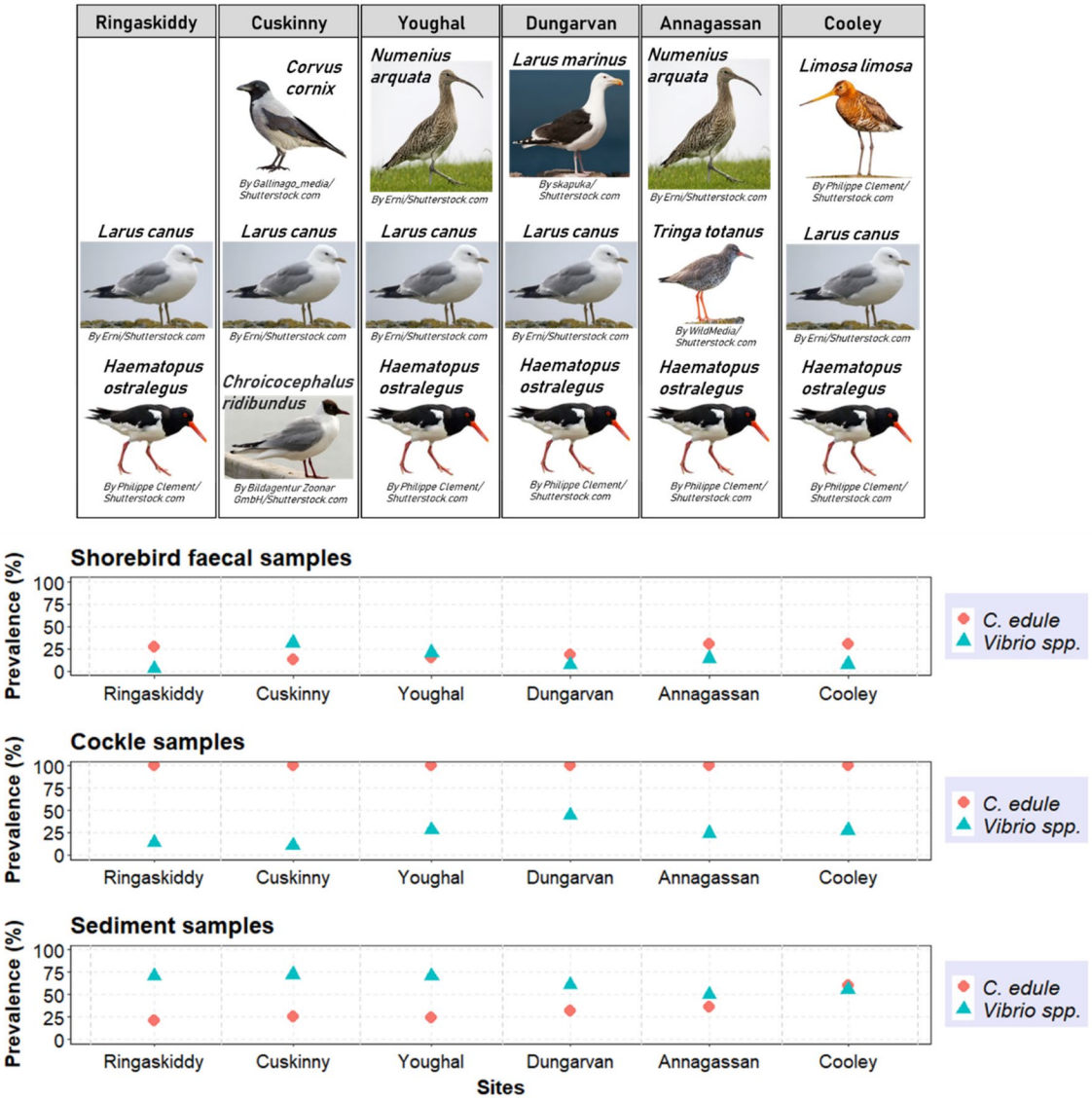

Figure 2. Distribution of *C. edule* DNA compared to the presence of *Vibrio* spp. in shorebird faecal samples, cockles, and sediment samples at each sample site. The most frequently observed shorebird species throughout the seasons at each sample site are represented on the top of the figure (Images used under license from Shutterstock.com).

The prevalence of *Vibrio* spp. displayed significant spatial variability ($p(Chisq) < 0.05$) in both *C. edule* and bird faecal samples (Fig. 2), although, no significant correlation ($p(\tau) > 0.05$) in the prevalence of *Vibrio* spp. was established between cockles and shorebird compartments. Ringaskiddy displayed the lowest prevalence of *Vibrio* spp. (2.9%; n = 1/34) in the bird faecal samples, but also showed a low prevalence of *Vibrio* spp. in *C. edule* samples (13.7%; n = 16/117) (Fig. 2). In turn, Youghal, Annagassan and Cooley showed intermediate values in the prevalence of *Vibrio* spp. detected in *C. edule* samples and bird faecal samples (Fig. 2). Cuskinny was the site showing the lowest prevalence of *Vibrio* spp. in cockle samples (10.7%; n = 15/140), while Dungarvan presented the highest prevalence (44.4%; n = 75/169) (Fig. 2). In contrast, the highest prevalence of *Vibrio* spp. detected in faecal samples was in Cuskinny (31.3%; n = 10/32), with the lowest level of *C. edule* DNA found in the faeces of that area (12.5%; n = 4/32) (Fig. 2); while Dungarvan was the second site with the lowest prevalence of *Vibrio* spp. in faecal samples (7.1%; n = 2/28) and a low level of *C. edule* DNA found in the faeces (17.9%; n = 5/28) (Fig. 2). No significant correlation ($p(\tau) > 0.05$) was established between the presence of *C. edule* DNA and *Vibrio* DNA detected within the shorebird compartment.

The presence of *Vibrio* spp. in sediment samples overpassed the prevalence of *Vibrio* spp. in bird faecal samples and to a lesser extent in *C. edule* samples (Fig. 2). The examination of collinearity in the prevalence of *Vibrio* spp. between the different compartments—sediment, cockles, and shorebirds – revealed a correlation ($\tau > 0.3$) between sediment and cockles.

In sediments samples, no significant differences in *Vibrio* presence between sample sites were displayed ($p(Chisq) > 0.05$). The occurrence of *Vibrio* spp. remained fairly high through the sites, regardless of the different

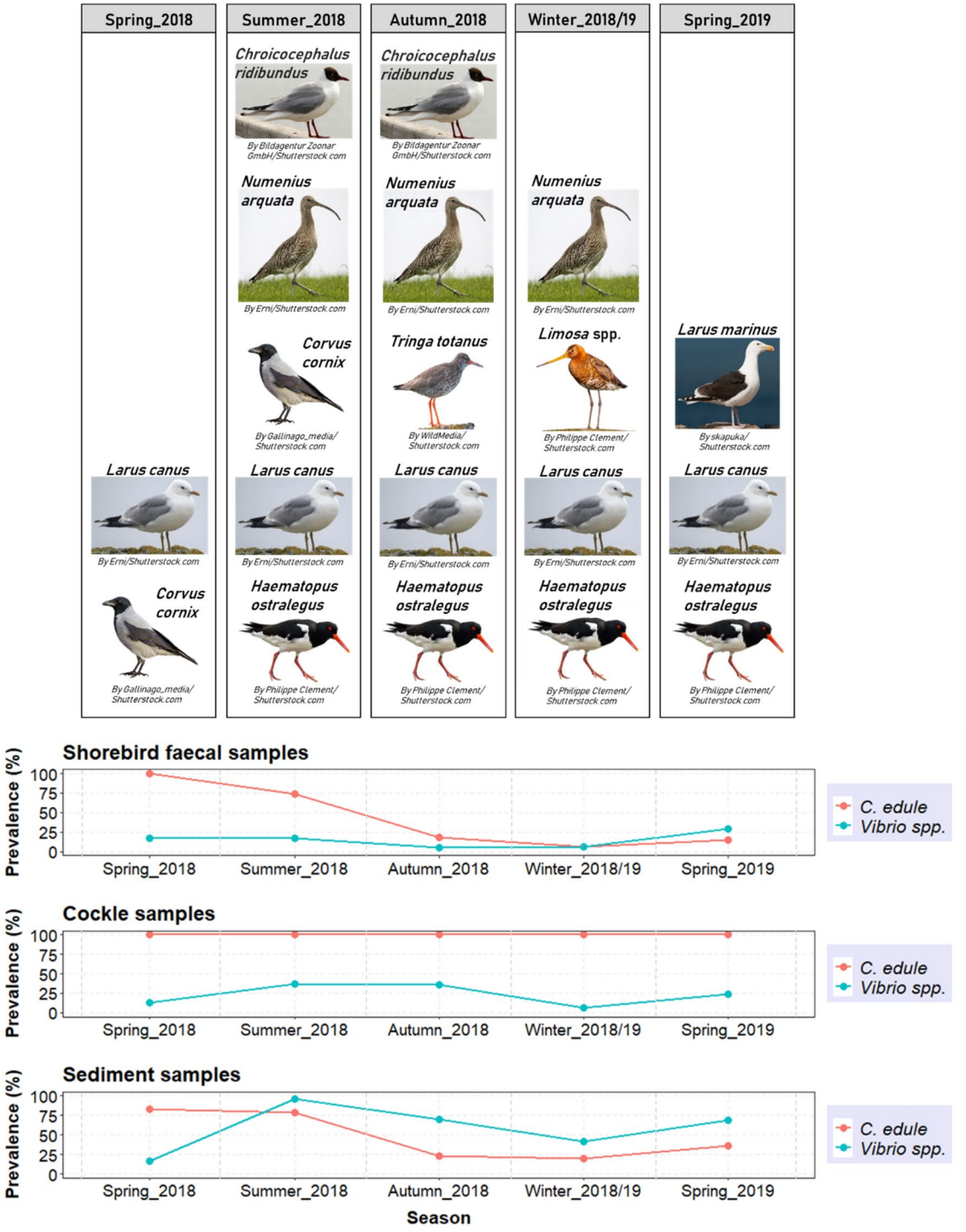

Figure 3. Distribution of *C. edule* DNA compared to the presence of *Vibrio* spp. in shorebird faecal samples, cockles, and sediment samples throughout the seasons. The most frequently observed shorebird species through the sites by each season are represented on the top of the figure (Images used under license from Shutterstock.com).

levels of *C. edule* DNA found in the sites—no significant correlation ($p(\tau) > 0.05$) was established between the presence of *C. edule* DNA and *Vibrio* DNA detected within the sediment compartment—(Fig. 2).

Significant seasonal variability ($p(Chisq) < 0.01$) in the occurrence of *Vibrio* spp. was exhibited in the three studied compartments—sediment, cockles, and shorebirds-. The highest presence of *Vibrio* spp. in *C. edule*, bird faecal samples and sediment samples, was during the warmer months and declined during the winter (Fig. 3). The presence of *Vibrio* spp., therefore, followed the same seasonal trend as the occurrence of *C. edule* DNA in both faeces and sediment samples (Fig. 3), although the correlation between *C. edule* DNA and *Vibrio* DNA detected

Pathogen species	Cockle samples	Seabird faecal samples	Sediment samples
Haplosporidia	**37.7% (277/735)**	0% (0/84)	0% (0/84)
Minchinia tapetis	**29.6% (82/277)**	0% (0/84)	0% (0/84)
Minchinia mercenariae-like	**14.8% (41/277)**	0% (0/84)	0% (0/84)
Ostreid herpesvirus type-1 and variants	0% (0/735)	0% (0/113)	0% (0/113)
Microsporidia sp.1	0% (0/735)	0% (0/58)	0% (0/58)
Microsporidia sp.2	0% (0/735)	0% (0/58)	0% (0/58)
Vibrio spp.	**25.3% (186/735)**	**13.7% (28/204)**	**62.7% (128/204)**
Vibrio aestuarianus	**19.9% (37/186)**	0% (0/204)	**1.6% (2/204)**

Table 2. Total numbers of positive cases (%, positive individuals/screened individuals) for the screening done for the target pathogen species in cockles, seabird faecal samples and sediment samples.

Sample type	Sample site	Season	Species identification	Percent Identity	Query Cover	Query Length (bp)
Cockles	Youghal Dungarvan Cooley	Summer 2018	*Vibrio spp.* among them: *Vibrio splendidus** and *V. kanaloae**	92.59–98.58%	78–83%	82–179
Cockles	Annagassan Annagassan	Summer 2018	*Vibrio spp.* most likely *V. splendidus**	97.84–97.92%	88–91%	154–155
Cockles	Annagassan	Summer 2018	*Vibrio spp.* most likely *V. aestuarianus* or *V. mediterranei*	98.01%	94%	158
Cockles	Cuskinny Dungarvan	Autumn 2018	*Vibrio spp.*	94.23–94.29%	67–73%	52–69
Sediment	Cooley Dungarvan	Summer 2018	*Vibrio spp.*	95.31–95.83%	85–93%	74–75
Sediment	Ringaskiddy	Autumn 2018	*Vibrio splendidus*	92.73%	85%	62
Sediment	Ringaskiddy Ringaskiddy	Autumn 2018 Winter 2018	*Vibrio sp.* most likely *V. splendidus**	95.65–98.51%	60%	110–114
Sediment	Youghal	Summer 2018	*Vibrio sp.* most likely *V. kanaloae**	94.37%	30%	222
Shorebird faeces	Dungarvan	Spring 2019	*Vibrio sp.* most likely *V. splendidus**	98.67%	86%	87
Shorebird faeces	Cooley	Summer 2018	*Grimontia hollisae (previously classified as Vibrio hollisae)*	97.14%	50%	138
Shorebird faeces	Youghal	Spring 2019	*Vibrionaceae bacterium*	100%	80%	57
Shorebird faeces	Annagassan	Autumn 2018	Enterobacter/Kiebsiella spp.	96.43%	73%	75
Shorebird faeces	Cuskinny	Autumn 2018	*Enterobacterales*	98.48%	44%	146

Table 3. Description of the Blast results obtained from the sequenced DNA of cockles, seabird faeces and sediment samples using generic *Vibrio* primers. (*) Same strains of *Vibrio* spp. were identified in cockle samples and sediment/faecal samples.

in those compartments was not statistically significant ($p(\tau) > 0.05$). 28.6% (n = 8/28) of the faecal samples that were positive for *Vibrio* spp. also contained *C. edule* DNA and all of them were from spring and summer. It was also noticeable that in spring 2019 the prevalence of infection was higher in the three compartments compared to spring 2018 (Fig. 3) when the only samples available were from Ringaskiddy and Cuskinny.

Discussion

This study demonstrates connectivity between the presence of vibrios in the marine environment and the different compartments that they can be detected in, i.e. sediment, invertebrates, and vertebrates. The presence of *Vibrio* spp. was confirmed in sediment samples, that may act as a reservoir or sink for such bacteria; in sediment-dwellers *C. edule*, where some may be pathogenic; and in shorebirds, infected via the consumption of cockles, resulting in transmission of these bacteria via faeces and the further repopulating of the sediment by these faecal bacteria. Unlike *Vibrio* spp., haplosporidians, including *Minchinia* spp., were found in *C. edule* samples but no transmission to shorebirds was detected. Haplosporidia DNA was also absent in the sediment. The lack of detection of haplosporidians supports the effectiveness of the *Vibrio* pathogen group to be transferred through the bird digestive system and persist in the environment. This finding may provide new insight into the life cycle of haplosporidians, which is still not fully known, and its capacity of persistence outside the host. However. further research in the infective stages of these pathogens is needed.

Two *Vibrio* species were identified that were of interest due to their pathogenicity and their major role in bivalve disease outbreaks[20,59–62]; *Vibrio splendidus*, known as an important aetiological agent of diseases affecting *Crassostrea gigas* aquaculture[60,61], and *Vibrio aestuarianus*, whose harmful effect has been reported on *C. edule*[59] and *C. gigas*[63]. For the first time, to the best of our knowledge, identical strains of *V. splendidus* have been reported in *C. edule,* faecal samples of wild shorebirds and sediment. *V. aestuarianus* was detected in *C. edule* through the sample sites and in sediment from Cuskinny and Dungarvan, but it was absent in the faecal samples. A potential explanation for this absence is that the bacterial load in cockles was not high enough to be transmitted to the birds during foraging or to be detected due to the low levels present.

V. splendidus detection in bird faeces further supports the carrier role that migratory birds play in the dissemination of *Vibrio* species, such as in *V. cholerae*[64–67], *V. parahaemolyticus, V. vulnificus* and *V. mimicus*[68–70]. It is well-known that the dispersal of *V. cholerae* by aquatic birds may be attributable to their direct predation of fish[71–73], chironomids and copepods[64]. Recently, Fu et al.[70] demonstrated that the predation of molluscs and zooplankton by migratory birds played an essential role in the dissemination of *V. parahaemolyticus* and *V. mimicus* in the estuary of the Liaohe River in China. However, the transmission of *Vibrio* species less relevant for humans had been neglected and very little is known about the role that migratory birds play in the spread of these pathogens. Our findings, therefore, provided the first evidence of possible transmission of *V. splendidus* to birds through consumption of infected cockles, as was supported by the presence of *C. edule* DNA and *Vibrio* DNA in the same bird faecal samples. This was further supported by the observations of waders, mainly oystercatchers *H. ostralegus*, known to feed on bivalves including cockles, at the sample sites. Cockle availability is a key resource supporting many overwintering wader populations such as oystercatchers[5]. The observational data collected also revealed that the presence of waders was larger at the sample sites with higher cockle abundances observed during the sampling. Previous studies have demonstrated a positive correlation between shorebird abundance and invertebrate prey densities, especially when patterns are examined across large spatial scales (e.g., encompassing entire estuaries)[74–76]. Moreover, the identified strains of *V. splendidus* in *C. edule* and bird faecal samples came from the same sample site, Dungarvan, with the highest prevalence of *Vibrio* spp. in *C. edule* samples.

The spatial variability in the *Vibrio* infection established in *C. edule* and bird faecal samples added evidence to the influence that cockle consumption may have had on the *Vibrio* prevalence detected in shorebird faecal samples. Wader flocks were common in Dundalk (Annagassan and Cooley) with the highest presence of cockles observed in the field and the highest densities reported in the literature (Shellfish Stocks and Fisheries Review, Marine Institute and BIM, 2017). Both sites showed the highest presence of *C. edule* DNA in the bird faeces, suggesting the waders are feeding on the cockles of those areas. In turn, Annagassan and Cooley showed intermediate values in the prevalence of *Vibrio* spp. detected in both *C. edule* samples and bird faecal samples. Despite the more frequent observation of waders in the sites with easier access to cockles, the correlation in *Vibrio* prevalence between *C. edule* and shorebird compartments was not significant. The strongest discrepancy in the prevalence of *Vibrio* spp. detected between the *C. edule* samples and the faecal samples was found at Cuskinny and Dungarvan. Dungarvan, with the highest prevalence of *Vibrio* spp. and *V. aestuarianus* in *C. edule*, showed a low prevalence of *Vibrio* spp. in bird faecal samples, probably due to the predominant presence of gulls, with a generalist diet, with respect to waders. Cuskinny exhibited the lowest prevalence of *Vibrio* spp. in cockle samples and the highest prevalence of *Vibrio* spp. in faecal samples, being also the gulls the dominant birds present. Therefore, it may reflect a lower consumption of cockles by gulls. This is consistent with the lower presence of *C. edule* DNA found in bird faecal samples at both sites. In the case of Cuskinny, the high prevalence of *Vibrio* spp. in faecal samples may be due to another source of *Vibrio* rather than the consumption of cockles.

Seasonal variability in the *Vibrio* infection was confirmed in *C. edule* and bird faecal samples and may also be related to the cockle consumption by shorebirds. Prevalence of *Vibrio* spp. increased in *C. edule* and faecal samples during the warmer months, probably due to the higher temperature. Temperature is likely the most important driver of the overall change in *Vibrio* abundance in temperate coastal waters[27]. Temperature promotes the proliferation of bacteria such as *Vibrio*, which grow preferentially in warm waters[26,27]. The high seawater temperature may also have an important impact on the physiological state of bivalve hosts, which makes them more susceptible to infection during the warm period of the year[77]. Consequently, infected *C. edule* may have been more accessible for shorebirds at that time of the year, when more screened cockles were found in poor condition. This higher susceptibility of cockles may have also promoted its opportunistic consumption by generalist feeders such as gulls and hooded crows, as suggested by the higher occurrence of *C. edule* DNA detected during the warmer months in the bird faecal samples. Although no significant correlation was established between the presence of *C. edule* DNA and *Vibrio* DNA in faecal samples, *Vibrio* prevalence followed the same seasonal trend as the occurrence of *C. edule* DNA in bird faeces. It was also noticeable that in spring 2019 the prevalence of infection was higher in *C. edule* and faecal samples compared to spring 2018. This might be because the only samples available in spring 2018 were from Ringaskiddy and Cuskinny, where the prevalence of *Vibrio* spp. was low in *C. edule* samples. Besides, in Cuskinny, gulls, which are not exclusively dependent on cockles for their feeding, were most frequently observed.

As expected, due to the infaunal lifestyle, *C. edule* DNA was also detected in sediment samples, with higher frequency than in the faecal samples. The detection of *C. edule* DNA in the sediment samples was higher at the sites with higher cockle abundances observed, i.e. Dundalk (Annagassan and Cooley). The detection of *C. edule* DNA in Ringaskiddy and Youghal was the lowest, as they were the areas where more effort was required to find enough cockles for the study. Empty cockle shells were observed at Ringaskiddy at every sampling, while Youghal is an extremely muddy shore, which makes it less suitable for cockles that prefer a sand-mud mixture best for cockle growth[5]. A significant correlation between the occurrence of *Vibrio* spp. in cockles and sediment was established, confirming the link between these two compartments. In sediment samples, the total frequency of *Vibrio* spp. detected was higher than in shorebird faeces and *C. edule* samples. Previous studies have shown that attachment to surfaces, such as the exoskeleton of chitin as well as sediment, is an integral part of the aquatic

lifestyle of many vibrios, representing a successful survival mechanism[27,28,78,79]. Vezzulli et al.[27] observed that both *V. aestuarianus* and *V. splendidus* maintained viability and culturability for longer times in the sediment than in seawater in laboratory experiments, suggesting that this compartment may represent a suitable niche for their persistence in the environment. Accordingly, Johnson et al.[11] observed a protective effect in sediment compared with oysters and seawater, for *V. parahaemolyticus* and *V. vulnificus*. Freitas et al.[80] recently demonstrated that *V. parahaemolyticus* cells attach to underwater surfaces, such as sediment, and/or associate with various species of shellfish and zooplankton as part of their life cycle. In our study, no significant spatial variability was detected in the presence of *Vibrio* spp. in sediment samples. *Vibrio* spp. frequency remained consistently high through the sample sites regardless of the different levels of *C. edule* DNA detected, with no correlation established between the *C. edule* DNA and *Vibrio* DNA found in the sediment. The findings highlight the role of the sediment as a major reservoir for *Vibrio* spp.

Vibrio spp. showed a lower prevalence during winter in both *C. edule* and faecal samples, however, *Vibrio* spp. frequency remained higher in the sediment. Accordingly, Vezzulli et al.[81] identified the sediment as an environmental reservoir for *Vibrio*, where the bacteria can find a favourable environment for overwintering. This is also probably linked to the fact that sediment provides biotic and abiotic surfaces useful for bacterial biofilm development; moreover, the concentration of organic matter in this compartment is higher than in the overlying water column (10,000–100,000 fold-higher in natural conditions)[81].

The strains of *V. splendidus* found in the sediment were the same as the ones in *C. edule* and bird faecal samples, although the strains of *V. splendidus* in sediment were identified in a different cockle bed, as well as the same strain of *V. aestuarianus* was detected in *C. edule* and sediment samples. In waterborne infections, in fact, the pathogenic agent can be shed by infected migrating birds, resulting in contamination of water with faeces or other corporal discharges[41]. Subsequently, molluscs inhabiting the area may get infected by filtration of the water column with the pathogenic agent in it[70]. The presence of pathogens in the sediment may also have an impact on the infaunal community within the sediment, which influence ecosystem services as bioturbation, nutrient recycling or carbon sequestration[5]. The presence of the same vibrios in the three analysed compartments expose, therefore, the intricate links of the marine trophic webs and the influence of the environment on them.

The findings obtained from this research provide new insight into the parasite-inclusive food web studies of mudflat ecosystems, targeting microparasites and pathogens that have received much less attention in this respect. The observed differences in the transmission of the Haplosporidia and the *Vibrio* spp. analysed indicates that not all pathogen groups are transmitted via food webs and that certain pathogens such as bacteria can be ingested and excreted with their DNA integrity intact thus indicating viability. The findings shed light on the potential transmission of *V. splendidus* associated with bivalves to shorebirds, via cockle consumption; as it has been previously reported with oysters[67] and fish[72], whose intake transmits *V. cholerae* to aquatic birds. Our findings add evidence to the significant role migratory birds may have as carriers of infectious agents such as bacteria, enabling their transport and dispersal not only to short-distance, given the efficient digestion and short food retention time in shorebirds, but also to long-distance if the infection in shorebirds occurs[82]. Further examination of regurgitated pellets and faecal droppings, as well as the analysis of gut contents of shorebirds, would be necessary to definitively confirm the subsequent trophic transmission and the infection success and impacts this pathogen can have on shorebird populations. Likewise, our findings further support the role of sediment as a *Vibrio* reservoir. The analysis of other compartments in the field, such as seawater, cohabiting bivalves, etc., and the factors affecting the survival of the infectious agents, would be of interest for future research to provide a complete picture of the infection sources and the potential connectivity between them.

Methods

Sample sites. Cockles, faecal samples and sediment samples were collected seasonally from April 2018 to April 2019 at Cork Harbour (Ringaskiddy and Cuskinny) on the south coast (Celtic Sea) of Ireland, and from July 2018 to April 2019 at Youghal Bay and Dungarvan Harbour, the latter the site of extensive farming of Pacific oysters (*Crassostrea gigas*), on the south coast (Celtic Sea), as well as at the commercial fishery at Dundalk Bay (Annagassan and Cooley) on the northeast coast (Irish Sea) (Fig. 4). The six locations are bays with intertidal sand and mudflats, where a variety of bivalve species inhabit. They 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). Moreover, Cuskinny is classified as Marsh Nature Reserve and Dundalk Bay is a classified Bivalve Mollusc Production Area and it has supported a commercial dredge cockle (*Cerastoderma edule*) fishery since 2001[83].

Although the cockle densities in situ were not measured, observations of cockle abundances during the samplings were in accordance with the reference densities previously recorded in those sites. *C. edule* densities in the selected sites varied from higher densities, 9.33 ± 3.5 ind/m^2, in Dundalk Bay (Shellfish Stocks and Fisheries Review, Marine Institute and BIM, 2017) to lower densities in sites that are not fished: 1.6 ± 2.1 ind/m^2 in Youghal Bay; 0.8 ± 1.7 ind/m^2 in Cork Harbour; and 0.4 ± 1.3 ind/m^2 in Dungarvan Harbour[13].

Bird observations. Video recording of shorebirds while foraging at low tide in the study areas was carried out seasonally from April 2018 to April 2019, following the specifications of Martins et al.[84]. Recordings were carried out with a digital camcorder (CX405B, Sony) with a 30× optical zoom. The video recording was done prior to sampling to avoid disturbance. The average duration of the films were 5 to 10 min, each one taken from a different perspective of the bay while filming the main bird aggrupations. Therefore, it is highly unlikely that pseudoreplication (i.e., filming the same individual more than once) affected our dataset and conclusions to any significant extent. The recording was done in the aerially exposed cockle bed, where the faecal samples and the sediment samples were collected at low tide. The viewing of the videos was carried out to list the bird species,

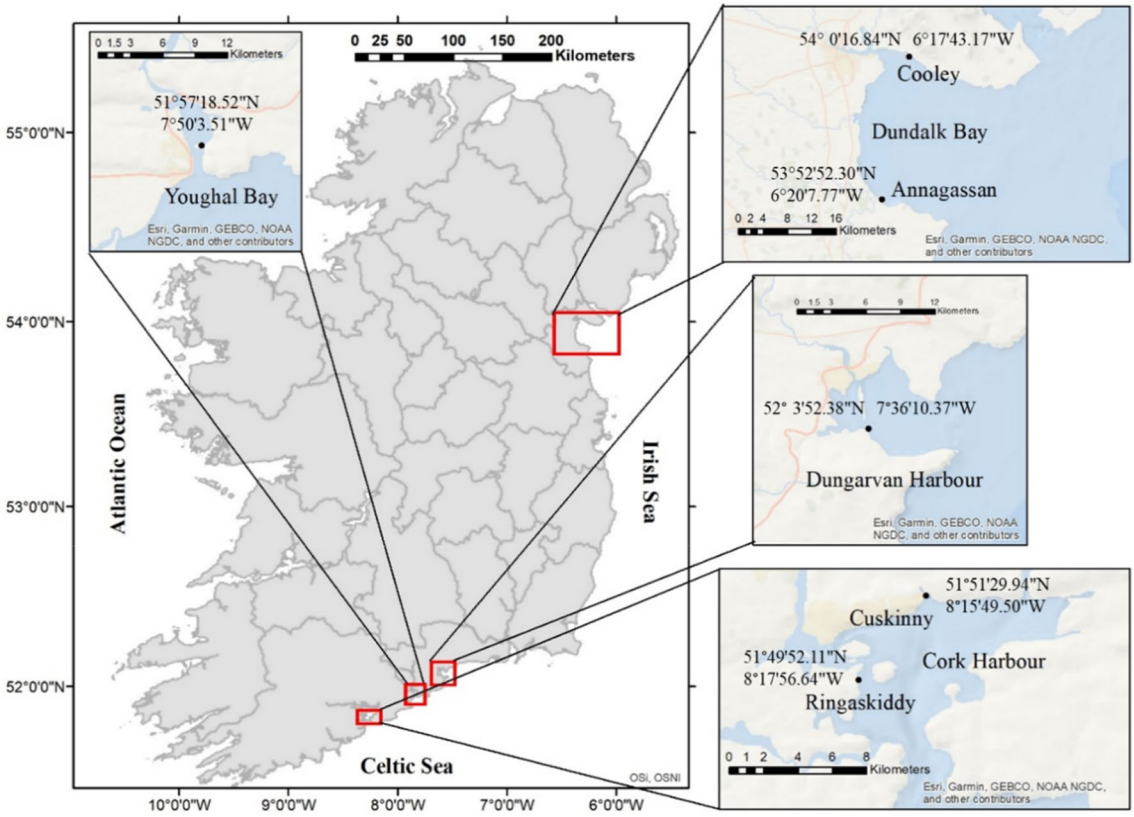

Figure 4. Map of Ireland highlighting the sample sites with coordinates (ArcGIS Desktop 10.5.1; www.esri.com).

	Cork Harbour—Ringaskiddy			Cork Harbour—Cuskinny			Youghal Bay			Dungarvan Harbour			Dundalk Bay – Annagassan			Dundalk Bay -Cooley		
Dates	***C***	***F***	***S***	***C***	***F***	***S***	***C***	***F***	***S***	***C***	***F***	***S***	***C***	***F***	***S***	***C***	***F***	***S***
Sp. 18	29	4	4	31	2	2	–	-	–	–	–	–	–	–	–	–	–	–
Sum. 18	12	–	–	27	–	–	11	4	4	51	3	3	43	6	6	17	10	10
Aut. 18	31	10	10	29	10	10	16	10	10	58	10	10	30	10	10	30	10	10
Win. 18/19	15	10	10	23	10	10	30	10	10	30	10	10	30	10	10	30	10	10
Sp. 19	30	10	10	30	10	10	12	10	10	30	5	5	30	10	10	30	10	10
Totals	117	34	34	140	32	32	69	34	34	169	28	28	133	36	36	107	40	40

Table 4. Number of collected cockles (C.), bird faecal samples (F.) and sediment samples (S.) by site and season (Sp.18: spring 2018; Sum.18: summer 2018; Aut.18: autumn 2018; Wint.18/19: winter 2018/19; Sp.19: spring 2019). (–) No samples collected.

their number and behaviour i.e., foraging and feeding or not, at the time of the filming. Following the descriptions in Lewis and Tierney[58], shorebird behaviour was classified in two categories: (1) foraging/feeding: the active or passive search of food and feeding; and (2) other: this category was used for all other behaviours that do not assign to foraging, including roosting, standing, preening, loafing, swimming and others. Prey identity was not examined. However, in some cases, analysis of video recordings allowed the distinction of the consumption of bivalves, even though identification of specific species could not be done even with the best quality recordings.

Sample types. Cockles were collected by hand raking from the intertidal area of each site at spring tide (0.5–1 m) and seasonal intervals. The sample size, outlined in Table 4, was dependant on the available abundance at each site and season. Cockles were kept at ambient temperature during the fieldwork and were processed either on the same day or were held overnight at 4 °C and were processed the next day.

Sediment and seabird faecal samples were collected as near as possible to the cockle collection area (Table 4). As food retention time in the digestive tracts of birds is short—for instance, for red knots (*Calidris canutus*) it is 20–50 min[82] -, the collection of samples was done after 30 min from the start of the video recording to ensure that the bird faeces collected were composed of food remains found and digested by the shorebirds feeding on

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*
Vib1-F Vib2-R	GGC GTA AAG CGC ATG CAG GT	GAA ATT CTA CCC CCC TCT ACA G	120	*Vibrio* spp.
HAP-F1 HAP-R3	GTT CTT TCW TGA TTC TAT GMA	AKR HRT TCC TWG TTC AAG AYG A	350	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
OHVA OHVB	TGC TGG CTG ATG TGA TGG CTT TGG	GGA TAT GGA GCT GCG GCG CT	385	Ostreid herpesvirus type-1 and variants
MicIF1 MicIR1	GTG GAC GCT AGT CTC ACA GGT T	TTG CAC CAG AAG GTT TAC GAC ACA T	180	Microsporidia sp.1
MicIF2 MicIR2	ATG CAT GCG TAA GCG AAG CAG TTA T	TCT CTT GCA CCA GAA GGT TTA CGA C	180	Microsporidia sp.2

Table 5. Description of PCR primer pairs showing sequences for each forward and reverse primer and expected product size.

prey from that foraging site[84]. Collection of samples was conducted following stringent contamination control strategies, such as using sterilised material and avoiding taking any substrate in contact with the faecal matter. One sterilised swab was used to collect a small amount (around 5 mm^3) of the bird faecal waste and a second swab was used to collect the same amount of sediment nearby to the stool. Each sample was transferred into an individually labelled 2 ml sterile Eppendorf tube.

Wherever possible, faeces were collected fresh to minimize further enzymatic action and, consequently, degradation of the DNA, although freshness is not always easy to determine in the field[52]. Preservation of faecal samples has mainly been by freezing in a range from −20 °C to −80 °C[53–56]. Seabird faeces and the sediment samples were stored in an insulated bag with iced packs during the fieldwork and, once in the lab, they were frozen at −80 °C until their processing to accelerate freezing and halt DNA-destroying enzymatic processes.

Sample processing and diagnostic methods. 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. Genomic DNA from the cockle gill tissue was extracted using the chelex-100 extraction method[85,86]. The DNA of seabird faecal samples, as well as the sediment samples, were extracted by Qiagen QIAamp DNA Stool Kit, following the protocol of Zeale et al.[87], with modifications from Dr James Nicholls (https://www.protocols.io/view/dna-extraction-from-avian-faeces-stored-in-ethanol-ve6e3he) and Sarah Davies (*pers comm*). Negative controls during DNA extraction were included to screen for potential contamination by prey DNA between samples and provide a higher degree of confidence in the assay protocol. Once extracted, the samples were used to determine the European cockle species (*Cerastoderma edule* (Linnaeus, 1758) or *Cerastoderma glaucum* (Poiret, 1789)) present and for subsequent pathological screening.

The quantity of target DNA remaining after storage, extraction and purification of the DNA, affects the successful amplification of that DNA from faecal material[52]. It should be highlighted that faecal DNA is often degraded, the risk of contamination is high, and PCR inhibitors may likely be co-extracted[52]. Therefore, the quantity and the quality of the extracted DNA from seabird faeces and sediment samples were established using NanoDrop 1000 Spectrophotometer to avoid the diagnosis of false negatives due to low DNA quantity and/or DNA of poor quality. DNA purity was assessed by determining the ratio of spectrophotometric absorbance of each extracted sample at 260/280 nm (A260/A280 ratio; an indicator of protein or phenol contamination). Pure DNA extraction should have an A260/A280 ratio ~ 1.8, higher values indicate the presence of RNA along with DNA.

Polymerase chain reaction (PCR) was carried out in the cockle, seabird faecal and sediment samples to amplify the nuclear DNA markers ITS-for / ITS Ce-R / ITS Cg-R to differentiate between the presence of *C. edule*, *C. glaucum* species or hybrids[88] (Table 5). Amplification was conducted following the reaction mixture and thermocycling conditions, as well as the visualisation of the product, described in Albuixech-Martí et al.[35].

Standard PCR screening in cockle, seabird faecal and sediment samples was conducted using generic primers Vib1F/2R[89] (Table 5) following the protocol modified by Vezzulli et al.[90] (optimised by Davies, C. E.) for the detection of *Vibrio* genus. A total of 1 ml of DNA per individual was used in a total volume of 25 ml of the reaction mixture containing: 12.5 ml of Taq 2X Master Mix, 0.125 ml of for and reverse primers (100 mM) and 11.25 ml of ddH_2O. Cycling conditions consisted of an initial denaturation of the sample at 94 °C for 10 min followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 60 s and a final elongation at 72°c for 10 min. Electrophoresis of the amplification products was conducted in a 2% agarose gel.

Primers	Sequence	Specificity
dnaJ f420	GTGAAGGGACGGGTGCTAAG	*Vibrio aestuarianus*
dnaJ r456	CCATGACAAGTGCCACAAGTCT	
dnaJ p441 (probe)	FAM-AGGGCACGTCGGC-MGB	

Table 6. Description of qPCR primer pair and probe used in the study.

Standard PCR screening for haplosporidian detection was carried out in the cockle samples (n = 735), likewise in a subsample of seabird faecal and sediment samples (n = 168), using generic haplosporidian HAP-F1 and HAP-R3 primers that amplify small regions of the SSU rDNA of most haplosporidian parasites[91,92] (Table 5). Amplification was conducted following the reaction mixture and thermocycling conditions, as well as the visualisation of the product, described in Albuixech-Martí et al.[35].

Standard PCR screening for *Minchinia* spp. detection was carried out in the cockle samples that were positive for Haplosporidia by conventional PCR (n = 277), likewise in a subsample of seabird faecal and sediment samples (n = 168), using specific primers for *Minchinia tapetis* (TAP-For/Rev) and *Minchinia mercenariae*-like (MER-For/Rev)[35] (Table 5) in the appropriate pairings and in separate PCR reactions. Amplification was conducted following the reaction mixture and thermocycling conditions, as well as the visualisation of the product, described in Albuixech-Martí et al.[35].

Standard PCR screening for ostreid herpesvirus type-1 (OsHV-1) and variants was carried out in cockle samples (n = 735), likewise in a subsample of seabird faecal and sediment samples (n = 226), using specific primers OHVA/OHVB that amplify small products of the ORF4 gene in the OsHV-1 virus and its variants[93] (Table 5). Amplification was conducted following the reaction mixture and thermocycling conditions described in Lynch et al.[93]. Electrophoresis of the amplification products was conducted in a 2% agarose gel.

Standard PCR using specific primers (MicIF1-MicIR1 and MicIF2-MicIR2, Table 5) (Lynch, unpublished data confirmed to be specific to Microsporidia spp. by Sanger sequencing) to detect two unidentified microsporidian species was carried out in cockle samples (n = 735), likewise in a subsample of seabird faecal and sediment samples (n = 116). Both reaction mixture and thermocycling conditions were the same as used with the PCR for OsHV-1, as well as the visualisation of the product.

Real-time quantitative PCR (qPCR) for detection and quantification of *Vibrio aestuarianus* in the cockle, seabird faecal and sediment samples that were positive for generic *Vibrio* screening was performed using specific primers for detection of the molecular chaperone *dnaJ* gene, following the protocol of McCleary and Henshilwood[63] (Table 6). All samples were performed in triplicates using 5 µl of DNA. C_t values were used to determine real-time PCR quantification and detection limits, a tested sample was considered positive if its mean C_t value was below 37.

Direct Sanger sequencing was carried out on representative PCR products amplified from cockle samples, seabird faecal and sediment samples, using generic *Vibrio* primers to identify the species present in the samples. Genomic DNA from 10 selected cockle individuals and 11 faecal and sediment samples 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 (Source Bioscience). Each sequence was matched against the National Center for Biotechnology Information (NCBI) nucleotide database with BLASTn (Basic Local Alignment Search Tool), which finds regions of local similarity between sequences to identify and confirm the DNA being detected in the PCRs.

Statistical analyses. Statistical analysis was performed in R version 3.2.3. statistical software. *Vibrio* occurrence was modelled as pathogen presence/absence in each cockle, bird faecal sample and sediment sample. Pearson's Chi-squared tests were used to determine whether the presence of *Vibrio* spp. was significantly different ($p(Chisq) < 0.05$) among the sample sites and throughout the year for each of the sample types (cockles, bird faecal samples and sediment). Fisher's exact test was conducted when the frequencies of the pathogen occurrence in a site or season were < 4, to gain accuracy.

Collinearity between the different compartments—sediment, cockles, and shorebirds—was examined by pairs using non-parametric Kendall's Rank Correlation Tau (τ) with the *Vibrio* occurrence data in each compartment ($p(\tau) < 0.05$). The correlation ($p(\tau) < 0.05$) between the presence of the *C. edule* DNA and *Vibrio* DNA was also examined within two of the compartments, shorebirds and sediment.

Data availability

All data generated during this study are included in this article.

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Author contributions

Conceptualization and methodology: S.A.M., S.C.C., S.A.L.; formal analysis: S.A.M.; writing—original draft: S.A.M.; writing—review and editing: S.C.C., S.A.L.; supervision: S.C.C., S.A.L. All authors approved the final version of the manuscript before submission.

Competing interests

The authors declare no competing interests.

Additional information

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