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University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

National University of Ireland, Cork



Understanding and minimizing the impacts of host-pathogen-environment interactions in the Pacific oyster *Crassostrea gigas*

Babette Bookelaar BSc MSc

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

School of Biological, Earth and Environmental Sciences.

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Head of School	Prof. Andrew Wheeler
Supervisors	Prof. Sarah C. Culloty
	Dr. Sharon A. Lynch

Table of contents

Abstract

5

Chapter 1. General introduction and outline

Overview of the Irish Pacific oyster *Crassostrea gigas* industry and the oyster pathogens that are having an impact.

8

Chapter 2. The role of the intertidal mobile predator and scavenger the shore crab Carcinus maenas in transmission dynamics of the Pacific oyster pathogen ostreid herpesvirus-1 microVar

Transmission dynamics of ostreid herpesvirus-1 microvar between the Pacific oyster *Crassostrea gigas* and the shore crab *Carcinus maenas*.

36

Chapter 3. Host plasticity supports spread of an aquaculture introduced virus to an ecosystem engineer

The role of moribund edible cockle *Cerastoderma edule* as a carrier or reservoir of ostreid herpesvirus-1 microVar at Pacific oyster *Crassostrea gigas culture sites.*

68

Chapter 4. The spread of the oyster pathogen Vibrio aestuarianus from a "hotspot" of infection in the marine environment

Investigation of the spread of ostreid herpesvirus-1 microVar and *Vibrio aestuarianus* in invertebrate species, edible cockle *Cerastoderma edule,* shore crab *Carcinus maenas* and mussel *Mytilus spp.,* from a Pacific oyster *Crassostrea gigas* culture site.

103

Chapter 5. Evaluation of the impact of different shore heights on oyster culture, at a disease endemic culture site

The effects of shore height and air exposure on the performance (growth and survival) and pathogen development (ostreid herpesvirus-1 microVar and *Vibrio aestuarianus*) in the Pacific Oyster *Crassostrea gigas.*

138

Chapter 6. The effects of crude and refined algal derived antiviral biotherapeutics on ostreid herpesvirus development and Pacific oyster Crassostrea gigas performance

The immune boosting and antiviral effects of seaweed spp. exposure on Pacific Oysters *Crassostrea gigas.*

175

Chapter 7. Discussion and conclusions

A summation of this PhDs contribution to a better understanding of oyster pathogens:*Crassostrea gigas*:environment relationship and dynamics.

208

Acknowledgements

212

Conferences: Poster and oral presentations

214

Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Babette Bookelaar

Abstract

Globally, Pacific oysters *Crassostrea gigas* are experiencing significant mortalities especially in warmer summer months, as a result of a complex aetiology and a strong association with pathogens such as ostreid herpes virus-1 microVar (OsHV-1 µvar) and bacteria *Vibrio aestuarianus*. Such mortalities threaten the future growth and sustainability of this industry worldwide. A better understanding of how these pathogens and diseases are interacting with their host and the environment at culture sites is needed, and new management and practical husbandry techniques are desired. This three-year study investigated the host-pathogen-environment interplay of Pacific oysters by carrying out field trials at the two largest culture sites in Ireland and by performing laboratory trials.

The objectives of this study were to:

(a) to understand how OsHV-1 µVar is becoming established at *C. gigas* culture sites. A five month field trial was undertaken in the spring and summer 2015. Green shore crabs *Carcinus maenas* and edible cockles *Cerastoderma edule* were collected at the oyster trestles and 500 m on the high shore at two culture sites, and were screened for OsHV-1 µVar by polymerase chain reaction (PCR), quantitative PCR (qPCR) and *in situ* hybridisation (ISH). OsHV-1 µVar was detected in both species by all diagnostics and was confirmed by direct sequencing. The mean prevalence of infection was 18.3% (n = 121/660) in Dungarvan and 16.3% (n = 155/952) in Carlingford Lough in *C. maenas* and 14.4% (n = 72/500) in Dungarvan and 13.6% (n = 121/890) in Carlingford Lough in *C. edule*. Following on from the field screening results, several 14 day laboratory trials were conducted to determine if naturally exposed crabs and cockles from culture sites could transmit OsHV-1 µvar to naïve oysters. OsHV-1 µvar was first detected in oysters cohabiting with exposed crabs on Day 4 and in oysters exposed to the cockles on Day 5. An overall prevalence of 6.5% in oysters held with crabs was observed while 4.4% was observed in the oysters held with cockles. Viral copies remained low (oysters exposed to crabs had an average of 1.0×10^2 viral copies μl^{-1} of genomic DNA, oysters exposed to cockles had an average of 3.0×10^3 DNA viral copies μl^{-1} of genomic DNA) in both trials, but this could be the result of trials

being run at a low temperature (14 °C) less than the threshold temperature (16 °C) for replication of the virus. To the best of our knowledge *C. maenas* is the first non-bivalve species indicating infection of OsHV-1 µvar.

(b) investigate the role of crabs, cockles and mussels as carriers or reservoirs for *Vibrio spp*.. In Ireland, *Vibrio aestuarianus* has been associated with *C. gigas* mortalities. *V. aestuarianus* was found at low infection intensities in *C. maenas* and *C. edule* and only at one of the main culture sites in 2015. Mussels *Mytilus* spp. were sampled from culture (2 hot spots) and non culture sites (<30 km from a culture site) in July, September and October 2017. OsHV-1 µvar was detected in *Mytilus spp.* in all samples screened and prevalence was significantly higher at the culture site. These results indicate that oyster pathogens and diseases are present in invertebrate species at *C. gigas* culture sites and extend beyond farms, even when prevalence in *C. gigas* is low.

(c) Determine the role of biotic and abiotic factors as drivers or inhibitors of pathogen and disease development in *C. gigas*. The effects of air exposure on pathogen development and oyster performance of different families and age classes over a period of 16-month period was investigated. Oyster seed and half-grown "Naïve" Irish and "Resistant" French bred stocks were held at the high shore and subtidally, approximately 6-8 hours difference in air exposure. Oysters were checked after 56 weeks (T1) and 68 weeks (T2) for mortality, growth and pathogen development (OsHV-1 µvar and *Vibrio aestuarianus*). A significantly higher prevalence of OsHV-1 µVar was detected in oysters 12 months after the trial commenced in June 2017 compared with oysters 16 months post relaying in September 2017, while this was not observed for *V. aestuarianus*, possibly suggesting a difference in disease development. No clear difference in disease development was observed between oysters located at high shore or lower shore, however especially the "Resistant" French stock performed better in survival and growth at the high shore. The "Resistant" French stock performed better in survival and growth compared to the "Naïve" Irish stock.

(d) Assess the effects of seaweed spp. commonly found attached to oyster bags on oyster health and pathogen development. Seaweed extracts, sulphated

polysaccharides, have shown to have a positive effect on the immune system of marine invertebrates by their wide variety of immune stimulating, anticancer, antiviral and antibacterial activities. In this study, the effects of natural degrading seaweed spp. on disease development in *C. gigas* seed when held under an increased temperature regime was investigated. Findings showed that exposure to seaweed spp. had a positive antiviral effect on OsHV-1 μVar development. The results from the testing of a commercial product of sulphated polysaccharides was inconclusive due to the lack of infection in all oysters.

Key findings

This research will not only give a better understanding of how the pathogens are maintained in the environment, it is also proposing possible consequences within a larger ecosystem perspective. Key findings from this study:

- Marine invertebrates, shore crab *Carcinus maenas* (Chapter 2) and edible cockle *Cerastoderma edule* (Chapter 3), commonly found at the intertidal area at culture and nonculture sites are carriers of OsHV-1 μVar.
- *Vibrio aestuarianus* was also detected in those two marine invertebrate species along with the blue mussel *Mytulis spp.*, even up to 30 km outside a hotspot of infection (Chapter 4).
- Shore holding height (difference in air exposure) of *C. gigas* can influence oyster performance and disease development for different age classes and oyster families (Chapter 5).
- Laboratory experiments indicated possible positive immune effects on seed oysters when exposed to seaweed species or algal derived commercial products (Chapter 6).

Chapter 1.
General introduction and outline

1.1. Pacific oysters *Crassostrea gigas*

The Pacific oyster, *Crassostrea gigas,* is distributed widely and in a range of environmental conditions, such as temperature ranging from -2°C to 35°C and salinity levels between 20 and 35 ppt (Ruesink et al. 2006; Smaal et al. 2009; Miossec et al. 2009). Due to its spread beyond its native range, by formation of reefs over native species, it has had widespread positive and negative impacts throughout its distribution. Globally, Pacific oysters are of ecological (Hosack 2003; Escapa et al. 2004; van der Zee et al. 2012) and commercial importance by functioning as an aquaculture species (FAO 2017). Pacific oysters, well known ecosystem engineers, can maintain, alter and/or create a physical environment due to their ability to create reef structures (Jones 1994; Breitburg et al. 2010). Natural oyster reefs facilitate other species by providing habitat and feeding grounds for other marine invertebrates and birds (Escapa et al. 2004; Van der Zee et al. 2012) and therefore may indirectly modify composition of benthic fauna (Eschweiler & Christensen 2011; Waser et al. 2015) and possibly higher trophic classes. In their introduced range, *C. gigas* can overtake native oyster species due to their rapid growth and high tolerance to a wide range of marine conditions (Ruesink et al. 2005; Ruesink et al. 2006; Smaal et al. 2009), while reefs of *C. gigas* also proved to reduce mortalities of certain native species (Waser et al. 2015) and even increase abundance of native epifaunal organisms (Escapa et al. 2004). Moreover, filter feeders like *C. gigas* can filter large quantities of water and therefore can alter benthic macro algal communities (van Leeuwen et al. 2010). Also, reef structures formed by *C. gigas* have been shown to change the environment physically by modifying the hydrodynamics (currents, sedimentation, erosion patterns) of the marine environment and therefore *C. gigas* may function as autogenic engineers (Walles et al. 2015).

In addition to their ecological importance, Pacific oysters are an important commercial aquaculture species worldwide (FAO 2017). The aquaculture industry expanded quickly and in 2014, with a global production of 625.925 tonnes, the

production of Pacific oysters was higher than any other aquaculture species (FAO 2017).

1.2. Status of the Pacific oyster industry in Ireland

Pacific oysters have been farmed in Ireland since the 1970's (Ó'Cinnéide et al. 2004; BIM / IFA oyster workshop on 20th March 2014). To date, Pacific oyster culture plays a significant role in Irish aquaculture production (Clegg et al. 2014) being the second largest segment of the Irish industry after caged salmon (European Commission 2014; BIM / IFA oyster workshop on 26th October 2016). The industry is growing, with production of almost 10.000 tonnes responsible for a production value of 41 million euro in 2016, which is an increase of 18% compared to 2015 (BIM 2017).

In 2016, 187 producers were involved in the Irish oyster industry (Annual NRL meeting on 28-30th of March 2017), providing 1300 jobs (BIM 2017). Production occurs in a total of 11 counties with counties Donegal and Waterford together being responsible for approximately 60% of the total production in Ireland. French producers account for a significant presence in the Irish oyster industry, responsible for a direct 25% of the total Irish oyster production. An additional 25% of the total Irish production is produced under contract for French interests (Renwick 2015). In addition, Irish oyster culture depends mainly on import of oyster seed and spat from France. The main culture method employed within the Irish Pacific oyster industry involves holding oysters in bags on trestles in intertidal areas, however a small percentage is cultured on the sea bed (European Food Safety Authority 2010). France is the major destination, accounting for 88% of the exported oysters. In addition, the Asian market is showing more interest and export has been increasing over the past few years (Renwick 2015).

Factors such as the ability to produce a top quality green food product, ambitious Irish oyster producers, an extensive coastline, favourable climate conditions for oyster culture and sustainable production systems gives Ireland a strong position within the European oyster industry. Therefore, the Irish oyster industry has opportunities to expand and make increasing economic profits into the near future (Renwick 2015). In 2012, an integrated Irish Marine plan 'Harnessing our Ocean

Wealth' financed by the Department of Agriculture, Food and the Marine set out an ambitious plan to support Blue Growth and Sustainable Food and Security (Harnessing our Ocean Wealth 2014). This plan forecasted a 78% increase in aquaculture volume production in Ireland by 2020. Nevertheless, there are a few challenges to the ambitious growth targets that the government and industry have set, including unresolved licensing issues, potential climate change impacts, variable seed supply and dependence on the French industry and continuing disease impacts that this study will focus on.

1.3. Mortality events in Pacific oysters worldwide

The number of records of abnormal mortality amongst Pacific oysters, *Crassostrea gigas,* worldwide has increased significantly since the 1950*s'* (Lynch et al. 2012) many, but not all, of these events have occurred during the summer months and have been denoted as 'summer mortality' events.

The cause of *C. gigas* summer mortalities are thought to have a complex aetiology, including interactions between the physical status of the host, environmental parameters (such as temperature, exposure time and salinity) and diseases and pathogens like ostreid herpes virus variants and/or *Vibrio* spp. bacteria (Burge et al. 2006; Samain & McCombie 2008; Sauvage et al. 2009; European Food Safety Authority 2010; Lynch et al. 2012). Since 1991, summer mortalities of young Pacific oysters have been regularly recorded and many have been associated with the pathogenic agent ostreid herpes virus 1 (OsHV-1). Ostreid herpesvirus 1 is part of the order Herpesvirales, family Malacoherpesviridae and genus Ostreavirus (Davison et al. 2009). New variants of OsHV-1, OsHV-1 var and OsHV-1 µVar, were initially identified and found to be associated with mortality outbreaks with up to 100 % in 2008 and 2009 (European Food Safety Authority 2010; Segarra et al. 2010; Dégremont 2013; Clegg et al. 2014). OsHV-1 µvar varies from OsHV-1 by a deletion of 12 base pairs in ORF 4 of the genome and proved to be more virulent (Segarra et al. 2010; European Food Safety Authority 2010).

1.4. Pathogens and diseases: Ostreid herpesvirus-1 microvar

Herpes viruses are characterized as K-selected viruses. They will co-exist with their hosts for extended periods and mortality will occur only sporadically, mainly

causing transmission, when environmental factors cross the threshold of the favourable range of the virus (Suttle 2007). Mortality outbreaks by OsHV-1 and its variants are associated with a sudden increase in water temperature or a water temperature above 16 degrees Celsius in Europe (European Food Safety Authority 2010; Clegg et al. 2014; Renault et al. 2014; Pernet et al. 2015). However, this threshold seems to be ecosystem specific as mortalities in Australia mainly have been observed when water temperature above 18 or 19 degrees Celsius (Evans et al. 2017). Viral strains of ostreid herpes 1 variants have been detected in all oyster stages, however mortality due to microvariants is mainly associated with spat and juveniles below 18 months (Arzul et al. 2002; European Food Safety Authority 2010). OsHV-1 µVar is described as a waterborne virus, with water playing a role as a medium for horizontal transmission (Schikorski et al. 2011) and therefore the virus is easily circulated in the marine system. Transmission of OsHV-1 therefore can take place horizontally from host to host or vertically from adults to offspring (Arzul et al. 2002; Barbosa-Solomieu et al. 2005) Oyster mortality is generally associated with and considered to be very likely when a viral copy number of 10^4 mg^{-1} oyster tissue or above is detected (Oden et al. 2011; Paul-Pont et al. 2013).

1.5. Pathogens and diseases: *Vibrio* species

More recently another pathogen, bacteria of the *Vibrio spp*. specifically *Vibrio splendidus* (Garnier et al. 2007; Saulnier et al. 2009; Saulnier et al. 2010) and *Vibrio aestuarianus* (Le Roux et al. 2002; Gay et al. 2004; Saulnier et al. 2010*)* have also been detected during mortalities of wild and cultured *Crassostrea gigas*. It has been suggested that co-infection with *V. splendidus* and *V. aestuarianus* could give synergistic virulence effects (Saulnier et al. 2010) which may also occur for *C. gigas* infected with both OsHV-1 and *V. splendidus* (Pernet et al. 2012) and OsHV-1 with *V. aestuarianus (*Azéma et al. 2016). Under experimental conditions, mortalities in *C. gigas* occurred after exposure to bacterial isolates of *V. aestuarianus (*Garnier et al. 2007; Saulnier et al. 2010; Azéma et al. 2017; Travers et al. 2017) and *V. splendidus* (Garnier et al. 2007; Saulnier et al. 2010; De Decker et al. 2011). Mortalities were also observed in the field, when wild *C. gigas* were naturally infected with *V. splendidus* and seawater temperature varied between 17 and 24 ºC (Pernet et al. 2012). However, the link between *Vibrio spp*. and summer mortalities in *C. gigas* has not definitively been determined, as other studies did

not indicate a clear pattern between prevalence of *V. splendidus* (Garnier et al. 2007; Saulnier et al. 2010; Pernet et al. 2012) and *V. aestuarianus* (Saulnier et al. 2010) and oyster summer mortalities. The significance of *Vibrio spp*. remains uncertain and those results suggest that the pathogen-host-environment interplay between *Vibrio spp.* and *C. gigas* may have a complex aetiology.

1.6. Mortality events in Pacific oysters in Ireland

In Ireland, summer mortalities, related to the presence of ostreid herpes virus, of *C. gigas* were first noted in 1993 (Clarke 1996; Cotter et al. 2010). In 2009 and 2010, significant summer mortality events, with up to 90% oyster mortalities, occurred in Ireland (Peeler et al. 2012; Cotter et al. 2010). In 2009 16 bays and in 2010 19 bays in Ireland associated with summer mortalities of *C. gigas,* reported the presence of strains of OsHV-1 µvar (Morrissey et al. 2015). Commencement of mortality occurred in all areas between mid-June to mid-July in 2009 and beginning of June to end of August in 2010 when sea water temperatures reached 15-16 °C and continued for one to five weeks. Mortality rates at the farms varied from 15–100% depending on location and affected stock. There was no detecetion of OsHV-1 µvar at seven Irish oyster farms that did not experience mortality (Morrissey et al. 2015). By 2012, herpes virus had spread to more culture sites around the country with a positive signal for OsHV-1 µVar detected in 29 bays (Morrissey et al. 2015). More recently in 2016, mortality events in *C. gigas* were lower than previous years with notifications in 4 oyster culture sites with spat mortality and 5 oyster culture sites with mortality in adults and / or half-grown oysters.

Besides OsHV-1 µvar, *Vibrio aestuarianus, Vibrio splendidus, Vibrio splendidus-like,* and *Vibrio gallicus* have all been detected in dying Pacific Oysters at Irish culture sites. Vibrio species have been isolated at fourteen out of fifteen Irish oyster farms with summer mortalities in 2009 (previously associated with OsHV-1 µVar infections): *V. splendidus* (13 bays), *V. splendidus-like* (4 bays), *V. aestuarianus* (5 bays) and *V. gallicus* (2 bays), levels of the bacteria reached high levels in 2013 (Marine Institute in European Food Safety Authority Journal 2010). Recent research conducted by the Marine Institute in Oranmore detected strains of *V. aestuarianus* in *C. gigas* in three of the four (Donegal Bay, Carlingford Lough,

Woodstown and Dungarvan) selected oyster culture bays. Mortality rates in one and two-year-old *C. gigas* at these three culture sites were linked with the presence of *V. aestuarianus,* while mortalities of younger stock were more often related to infection by OsHV-1 µVar. Mortality associated with *V. aestuarianus* mainly occurred in the autumn months during September and October and rates were significantly different between sites (Annual NRL meeting on 28-30th of March 2017). The forecasted growth of aquaculture production by Harnessing our Ocean Wealth is threatened by these high mortality rates.

1.7. The role of the environment in disease development in Pacific oysters

Pathogens OsHV-1 and variants and *Vibrio spp.* seems to play a key role in summer mortalities within Pacific oysters. Nevertheless, the development of the pathogens and diseases are associated with a complex aetiology including various physical, chemical, biological, and ecological interfaces. Hence, the environment and its elements play a significant role in disease transmission (Mydlarz et al. 2006; Dégremont 2011*)*, also identified as the 'pathogen–host–environment interplay' (Engering et al. 2013).

Environmental factors, hosts and/or other pathogens might function as risk factors for supporting viral infection of summer mortalities (European Food Safety Authority 2010). Moreover, annual or seasonal variations in a number of environmental parameters appear to play a significant role in oyster survival during mortality outbreaks (Cheney et al. 2000).

1.8. Abiotic stressors in Pacific oyster mortality events

Host:pathogen interactions may be impacted on by a range of abiotic environmental parameters, such as water temperature (Van Bannig 1997; Goulletquer et al. 1998), dissolved oxygen, eutrophication (Tamate et al. 1965; Malham et al. 2009), salinity levels (Luna-Gonzalez et al. 2008) and presence of pollutants (Bodoy et al. 1990; Cheney et al. 2000; Elanadaloussi et al. 2009; Moreau et al. 2015) which all may influence mortality levels in Pacific oysters.

Water temperature is known to be one of the main risk factors for increased mortalities in Pacific oysters related to ostreid herpes virus (Renault et al. 2014). Significant mortalities of *C. gigas* associated with OsHV-1 and variants are observed, particularly when seawater temperatures reach 16 °C and higher (Clegg et al. 2014; Renault et al. 2014; Pernet et al. 2015). Additionally, acute abnormal mortality has been observed associated with a sudden increase in water temperature (European Food Safety Authority 2010). This phenomenon is in agreement with summer mortalities detected in Ireland. In 2011 and 2012, which were comparatively cool summers, mortality associated with the virus was low. In 2009, 2010 and 2013 temperatures reached higher levels and mortality was higher (FSA Panel on Animal Health and Welfare (AHAW), 2015). A recent study observed an interruption of mortality when seawater temperature was higher than 24°C (Pernet et al. 2012), by decreasing the susceptibility of oysters to the virus (Pernet et al. 2012; Delisle et al. 2018). In addition, OsHV-1 transmission from OsHV-1 exposed *C. gigas* to naïve *C. gigas* under experimental conditions was observed when held at temperatures between 16.2 and 21.9°C, suggesting that this might be the optimal range for the virus (Petton et al. 2013).

Temperature might not only activate the virus, but it can also affect the energy balance of the oyster by influencing reproductive organs, nutrient availability and respiration rate (Samain 2011). Mass mortalities have been observed in the presence of high particulate organic matter (POM) or also seston (Chávez-Villalba et al. 2007). A high nutrient concentration in the water might favour fast growth rates and gonadal maturation and therefore indirectly introduce energy imbalance (Perdue et al. 1981) supporting natural infection of viral pathogens and diseases (Malham et al. 2009; Samain 2011) and/or mortalities (Perdue et al. 1981; Goulletquer et al. 1998). In addition, a relationship between dissolved oxygen and mortality rates of *C. gigas* has been observed in the field. In late summer, when phytoplankton blooms were observed, dissolved oxygen rates decreased, while mortality rates of *C. gigas* increased (Cheney et al. 2000). Also, variations in salinity did impact on survival rates of marine invertebrates (Gunter 1961; Jorge 1988, Gardner & Thompson 2001; Laing 2001). Eastern oyster, *Crassostrea virginica*, suffered massive oyster mortalities caused by parasite *Haplosporidium nelsoni* (MSX) and field studies found very low infections of MSX when oysters

were hold at salinities below 15 ppt, following the introduction of the successful management practice of holding *C. virginica* higher upstream in estuaries (Ford & Haskin 1988). Flannery et al (2014) found a similar reduction in *Bonamia ostreae* levels in *Ostrea edulis* when a screening programme took place in Clew Bay, Ireland. Several site in the bay with large freshwater inputs had negligible infection levels, compared to other beds with more usual saline conditions.

C. gigas is tolerant to a wide range of salinity levels; very low salinities below 10 ppt. up to 35 ppt. (Helm 2005). A recent laboratory study performed multiple salinity trials with Pacific oysters which where naturally exposed to OsHV-1 µVar and concluded that when oysters are infected, they have the highest survival rates at lower salinity levels (10 ppt) compared to higher salinity levels (15, 25 and 35 ppt). However, this pattern was only observed when oysters were acclimated to salinity, as non-acclimated oysters showed significantly lower survival rates, while having a similar infection level of OsHV-1. Therefore, a salinity shock might have diminished the impact of reduced viral replication and impact (Fuhrmann et al. 2016).

Mortality rates have also been shown to differ between culture methods (Soletchenik et al. 1999; Soletchenik et al. 2005). Studies demonstrated a relationship between proximity of the oysters to the bottom sediment and mortality (Samain et al. 2005; Royer et al. 2007), with lower mortalities in oysters held in the water column (Soletchenik et al. 1999; Soletchenik et al. 2005).

Generally Pacific oysters are cultured in estuaries or intertidal area and thus are exposed to the air for several hours a day, with exposure to variable factors such as temperature, oxygen levels and nutrient loads (Burnett 1997). Long air exposure time will reduce exposure to food, potentially resulting in lower growth rates, unfavourable energetic balance and make animals more susceptible to pathogen and diseases resulting in mortalities (Oesterling & Petrone 2012). The potential benefits of air exposure however might include controlling biofouling of hydroids, bryozoans, sea squirts, etc. growing on oyster trestle bag (Oesterling & Petrone 2012). OsHV-1 and its variants are transported through the water (Sauvage et al. 2009; Schikorski et al. 2011) and so less immersion time may lead to a lower

exposure to viral compounds (EFSA 2015). It is highly likely that length of air exposure is having some effect on performance and disease development in *C. gigas*.

Less is known about the effects of abiotic factors on *Vibrio spp.* activity, however bacterial activity is also associated with environmental parameters such as salinity, pH and nutrient availability (Hanson et al. 1986; Zdanowski & Figueiras 1999; Lee et al. 2001; Armada et al. 2003; Thompson et al. 2004; Figueiras et al. 2006; Romero et al. 2010). *V. aestuarianus* in specific was detected in higher bacterial loads in water, plankton and sediment during warmer periods and during periods of high primary production caused by upwelling events (Romero et al. 2010).

1.9. Biotic factors in mortality events in Pacific oysters

Within host:pathogen interactions in marine systems, a range of species and trophic interactions may play a role in disease transmission, with other animals potentially acting as carriers and reservoirs for pathogens (Lynch et al. 2007; Lynch et al. 2010; Small & Pagenkopp 2011; Carrasco et al. 2012). A carrier is seen as an incidental, asymptomatic host and a distributor of infection, while a reservoir can retain the pathogen permanently and transmit it back to the natural host (Haydon et al. 2002; Lynch et al. 2010).

It is uncertain if *C. gigas* functions as a single host (Arzul et al. 2001a) as OsHV-1 and variants have been detected in multiple different marine species in the past (Renault 1998; Renault et al. 2000; Arzul et al. 2001a; Arzul et al. 2001b; Renault et al. 2001) and recently also in eastern oyster *Crassostrea virginica* (Burge et al. 2011), Mediterranean mussel *Mytilus galloprovincialis* (Burge et al. 2011), the Sydney rock oyster *Saccostrea glomerata*, Sydney cockle *Anadara trapezia*, blue mussels *Mytilus spp.*, hairy mussel *Trichomya hirsuta*, whelks *Batillaria australis* and barnacles *Balanus spp*. (Evans et al. 2017). Whether these species are incidental hosts or carriers or reservoirs has not been definitively determined. However to fully understand the disease, there remains a lot of uncertainty about aspects of the pathology and ecology: how long can oyster pathogens sustain itself outside the oyster; do other marine species function as carriers, reservoirs or

alternative hosts and how might this explain disease dynamics; any observed wider ecological effects and how might it impact on the management of the disease.

In a recent EU collaborative project (BIVALIFE) conducted at UCC and with the Marine Institute, Ireland as the other Irish partner, the influence of varying environmental parameters on OsHV-1 µVar development (promotion and inhibition) and the ability of the virus to sustain itself and survive in the environment in Ireland was investigated. Preliminary studies indicated that the virus can be detected in the environment, mainly in plankton samples in the water column and biofilm samples. Also, other marine invertebrates, like bivalves (Blue mussels *Mytilus edulis* and edible cockle *Cerastoderma edule*), gastropods (*Nucella lapillus* and *Littorina littorea*), tunicates, crustaceans (Green shore crab *Carcinus maenas*), polychaetes and some chordates showed prevalence of OsHV-1 µVar at Pacific oyster culture sites in Ireland. Additionally, macroinvertebrates showed prevalence of *V. splendidus*, all year around, at the same Pacific oyster culture sites (Bivalife 2014). The role of these species in viral replication and transmission has not been determined. Of interest, a recent study found OsHV-1 µVar infected wild *Mytilus spp.* at *C. gigas* culture sites and also at non-culture sites in Ireland. Under an elevated temperature regime, viral transmission between wild *Mytilus spp.* and naïve *C. gigas* was observed in the laboratory after 14 days (O'Reilly et al. 2017). These results demonstrated that other marine invertebrates could function as possible carriers, reservoirs or even alternative hosts of these oyster pathogens. From an industry perspective, but also from an ecological, a fuller understanding of pathogen (OsHV-1 µVar and *Vibrio aestuarianus)* transmission dynamics in the marine ecosystem will provide a better perspective on the disease dynamics and potential control strategies that might be effective. Therefore, in this study, the role of marine invertebrates; the ecosystem engineer the edible cockle *Cerastoderma edule* and the mobile predator and scavenger the green shore crab *Carcinus maenas* were investigated. These two species are often found around oyster culture sites and have varying roles in the intertidal ecosystem. Following up, the presence of *Vibrio aestuarianus* in these species and in mussels *Mytilus* spp. the blue mussel *M. edulis*, the Mediterannean mussel *M. galloprovincialis* and their hybrids was determined to answer similar questions for this bacteria. Next, the potential for spread of these pathogens from culture sites

was determined. Potential spread of these pathogens to the wider ecosystem could potentially have a widespread impact at different trophic levels and disrupt the community structure.

1.10. Management and husbandry techniques to support the Pacific oyster industry

The culture of marine species in the open water makes the eradication of their pathogens and diseases almost impossible, as host and carriers cannot be fully removed and full life cycles of many pathogens are unclear (Peeler & Otte 2014). Movement legalisation was introduced in 2010, where Ireland, Spain, the Netherlands and the United Kingdom agreed on movement restrictions, via Commission Regulation (EU) No 175/2010, to control increased mortality of Pacific oysters and to prevent the further spread of OsHV-1 µvar (www[1]). Where the virus has already been introduced, control mechanisms have to focus on methods to reduce the impact and spread of disease. Management tools like vaccination (Gestal et al. 2008; Green & Montagani 2013), selective breeding programs for pathogen resistant species (Dégremont 2011), movement legislations (Renault 2011) and removal of host species (Ben-Horin et al. 2016) have been suggested but are in need of closed systems, are labour intensive, time consuming or very expensive (Peeler & Otte 2014).

Selective breeding programs in France were set up to produce a new generation of Pacific oysters which are resistant to OsHV-1 µVar to improve oyster survival in unfavourable circumstances (Dégremont 2011). Dégremont showed significantly higher resistance to summer mortality in selected spat, juvenile and adult oysters compared with the control batch, which were sourced from an unselected wild population (Dégremont 2011; Dégremont et al. 2015). More recent research also demonstrated lower viral copies of OsHV-1 µVar in selected larvae, resulting in lower mortalities compared with unselected larvae and therefore proving genetic resistance to OsHV-1 infection at the larval stage (Dégremont et al. 2016). Previously, a breeding program to reduce mortality rates associated with marine pathogens has proven to be successful in European flat oyster *Ostrea edulis* and the protistan pathogen *Bonamia ostrea*, with low mortalities and prevalence of the parasite being observed (Lynch et al. 2014). There is no evidence to date to

indicate that a breeding programme to selectively breed against *Vibrio aestuarianus* or *Vibrio splendidus* has been developed.

While certain management practices can be put into place, variable environmental factors like temperature, nutrient load, dissolved oxygen and salinity can still impact on development of disease. However, new practical measures and husbandry techniques could support oyster growth and reduce risk of pathogen development by taking into account favourable environmental conditions that minimise development of disease. Therefore, in this study, the effects of air exposure on performance and disease development of Pacific oysters was investigated by holding oysters at different shore heights in the field. Next, the role of crude and refined algal derived antiviral bio-therapeutics were tested for performance and pathogen development of naïve and pathogen exposed oysters. This could, support new measures or practical husbandry techniques to maintain or even increase production of the Irish oyster industry.

1.11. REPOSUS: REducing the impact of Pathogens and disease in the Irish Oyster industry to support the SUStainability and growth of the sector

This PhD was part of the REPOSUS Project "Reducing the impact of Pathogens and disease in the Irish Oyster industry to support the sustainability and growth of the sector" funded under FIRM by Department of Agriculture, Food and the Marine (DAFM) 14 SF 820. This was a collaborative three- and a half year multidisciplinary project, between University College Cork and the Marine Institute started in April 2015. The aim of the project was to gain a better understanding of some of the drivers of the pathogen-host-environment interaction in the Pacific oyster in Ireland, to come up with new management tools and husbandry techniques to support the health of the Pacific oysters and to enhance and support the Irish oyster industry.

1.12. Outline of the thesis

The main research focus of this study was to understand and to minimize the impacts of host-pathogen-environment interactions in the Pacific oyster *Crassostrea gigas*.

In chapter 2 and chapter 3, field experiments were taken place to investigat how OsHV-1 µVar is sustaining itself in the environment outside its host *C. gigas* by the potential of the shore crab *Carcinus maenas* and edible cockle *Cerastoderma edule* to act as a carrier or reservoir of OsHV-1 µVar. Additionally, tit was studied if those invertebrates could function as reservoirs OsHV-1 µVar. Thereforet ransmission dynamics of ostreid herpes virus between naïve *C. gigas* and naturally virus exposed *C. maenas* and *C. edule* were studied under experimental conditions.

In chapter 4, the extent to which *Vibrio aestuarianus* is present outside the oyster was investigated in crabs, cockles and mussels. In addition, the geographical extension range of oyster pathogen *V. aestuarianus* extending out from two hotspots of infection at culture sites was studied.

In chapter 5, the role of air exposure on pathogen development and Pacific oyster performance was investigated. A one-and-a-half-year study was performed in the field at an oyster culture site with a history of herpes virus and *Vibrio aestuarianus* by exposing two different age classes (seed and half-grown) and two different *C. gigas* families (French “Resistant” bred stock and Irish “Naïve” stock) at different air exposure circumstances with maximal air exposure at high shore and minimal air exposure at low shore.

In chapter 6, the possible role of antiviral compounds on pathogen development and oyster performance was investigated. Seaweed extracts, sulphated polysaccharides, have been shown to support the immune system of marine invertebrates by their wide variety of immune stimulating, anticancer, antiviral and antibacterial activities. To determine if these compounds could reduce the impact of pathogen on the Pacific oyster the role of seaweeds on pathogen development and oyster performance was investigated under laboratory conditions.

In Chapter 7, a general discussion is presented, including recommendations and guidelines to support the growth and sustainability of the Pacific oyster *Crassostrea gigas* sector.

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Chapter 2.

The role of the intertidal mobile predator and scavenger the shore crab *Carcinus maenas* in transmission dynamics of the Pacific oyster pathogen ostreid herpesvirus-1 microVar

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B. E. Bookelaar, A. J. O'Reilly, S. A. Lynch, S. C. Culloty,

Aquaculture and Fisheries Development Centre, School of Biological, Earth and Environmental Sciences &

Environmental Research Institute, University College Cork, Cork, Ireland

Corresponding author: b.bookelaar@umail.ucc.ie

Abstract

Ostreid herpesvirus-1 microVar (OsHV-1 μVar) has been responsible for significant mortalities globally in the Pacific oyster, *Crassostrea gigas*. While the impact of this virus on the Pacific oyster has been significant, this pathogen may have wider ecosystem consequences. It has not been definitively determined how the virus is sustaining itself in the marine environment and whether other species are susceptible. *Carcinus maenas* is a mobile predator and scavenger of *C. gigas*, commonly found at Pacific oyster culture sites. The aim of this study was to investigate the role of the crab in viral maintenance and transmission to the Pacific oyster. A field trial took place at different shore heights at two Irish Pacific oyster culture sites, over a summer, that are endemic for OsHV-1 μVar. Infection of OsHV-1 μVar in tissues of *C. maenas* at both shore heights of both sites was detected by polymerase chain reaction (PCR), quantitative PCR (qPCR), *in situ* hybridization and direct Sanger sequencing. In addition, a laboratory trial demonstrated that transmission of the virus could occur to naïve *C. gigas* within four days, from *C. maenas* previously exposed to the virus in the wild. These findings provide some insight into the possibility that the virus can be transmitted through marine food webs and suggests viral plasticity in the hosts required by the virus and potential impacts on a range of crustacean species with wider ecosystem

impacts if transmission to other species occurs.

Key words

Crassostrea gigas, Carcinus maenas, ostreid herpesvirus-1 microvar, pathogen–host–environment interplay, predator-prey, scavenger

2.1. Introduction

Diseases, parasites and pathogens are common in marine ecosystems (Lafferty et al. 2015) and have a significant impact on fisheries and aquaculture (Willman et al. 2009; Lafferty et al. 2015), as well as the ecology of marine habitats (Harvell et al. 2002). Development of disease is in general due to a complex aetiology including numerous physical, chemical, biological, and ecological interactions. Hence, the environment and its constituents play a significant role in disease transmission (Mydlarz et al. 2006; Degremont 2011*),* also known as the 'pathogen–host–environment interplay' (Engering et al. 2013).

Virus infections in bivalve species have been associated with high mortality rates, when conditions become less favorable for the host species (Rowley et al. 2014). A significant pathogen-host-environment interplay has been observed for the commercially important Pacific oyster *Crassostrea gigas* with ostreid herpesvirus (OsHV-1) and variants, which has resulted in mass mortalities among early life stages of *C. gigas* worldwide (Burge et al. 2007; Lynch et al. 2012; Prado-Alvarez et al. 2016*).* In particular, nowadays these mortalities have been associated with the variant OsHV-1 microVar (OsHV-1 µVar), which is considered highly virulent (Segarra et al. 2010) especially when seawater temperatures reach 16°C and higher (Clegg et al. 2014; Renault et al. 2014; Pernet et al. 2015). The virus has already been proven to be waterborne in previous studies (Vigneron et al. 2004; Sauvage et al. 2010; Schikorski et al. 2011; Evans et al. 2015). Infected adult oysters may function as carriers and infect naïve spat by vertical transmission (Arzul et al. 2002; Barbosa-Solmieu et al. 2005) and horizontal transmission between healthy and experimental infected oysters has been observed (Schikorski et al. 2011).

Viral transmission within the marine environment provides a medium that can expose all animals within that habitat to a source of infection. Whether viral transmission occurs solely from primary host to primary host is a key point in understanding those dynamics. However, in other host:pathogen interactions in marine systems a range of species and trophic interactions may play a role in disease transmission, with other animals acting as carriers and reservoirs for pathogens (Lynch et al. 2007; Lynch et al. 2010; Small & Pagenkopp 2011). Carriers or reservoirs have been defined as species that can function as a source of infection. A carrier is seen as an incidental, asymptomatic host and a distributor of infection, while a reservoir can retain the pathogen permanently and transmit it back to the natural host (Haydon et al. 2002; Lynch et al. 2010). Furthermore, in specific scenarios, pathogens and diseases can change their host range by selecting new target species as an alternative host (Howard & Fletcher 2012; Engering et al. 2013; Schrauwen & Fouchier 2014).

Infectious disease outbreaks can occur when carrier species, mostly "non-pathogenic" for the specific pathogen, come in contact with a susceptible host species (Burek et al. 2008). It is important to note that viruses are able to jump host as they have been shown to demonstrate plasticity and rapid evolution in terms of hosts targeted, allowing them to respond to and infect a range of potential hosts in new habitats (Johnson et al. 2015; Geoghegan et al. 2017). It is uncertain if *C. gigas* functions as a single host (Arzul et al. 2001a) as herpes-like virus have been detected in multiple different marine species in the past (Renault 1998; Renault et al. 2000; Arzul et al. 2001a; Arzul et al. 2001b; Renault 2001) and recently also in invertebrates such as the oyster *Crassostrea virginica* (Burge et al. 2011), Mediterranean mussel *Mytilus galloprovincialis* (Burge et al. 2011) and Chinese scallop *Chlamys farreri* (Ren et al. 2013). More recently OsHV-1 µVar was detected in the Sydney rock oyster *Saccostrea glomerata*, Sydney cockle *Anadara trapezia*, blue mussels *Mytilus spp.*, hairy mussel *Trichomya hirsuta*, whelks *Batillaria australis* and barnacles *Balanus spp*. (Evans et al. 2017). For most invertebrate species other than oysters infected with herpes-like virus the pathogenic effect is still unknown, however Chinese scallop *Chlamys farreri* suffered mass mortality after infection (Ren et al. 2013) highlighting the potential impact of this virus on its marine environment.

The intertidal zone where *C. gigas* are cultured on trestles contains a range of sessile and mobile filter feeders, scavengers and predators. The European shore crab *Carcinus maenas* is native to the Atlantic coasts of Europe and Northern Africa and is invasive on the west coast of North America, South Africa, Australia and Tasmania (Torchin et al. 2001; Carlton & Cohen 2003). Outside its natural range, *C. maenas* has often been seen as a pest (Lafferty & Kuris 1996) by causing significant ecological and evolutionary impacts, such as altering community structures (Torchin et al. 2002) and by reducing densities of different species of taxa including bivalves, cumaceans and amphipods (Grosholz & Ruiz 1995). *C. maenas* is common at estuarine intertidal habitats (Amaral & Paula 2007) and feeds upon a diverse variety of prey including commercially important species blue mussel *Mytilus edulis* and Pacific oyster seed and juveniles (Lovely et al. 2015). *C. maenas* are known to be attracted to oyster trestles both as a food source and for protection from predation (Lovely et al. 2015). Of significance, *C. maenas* acts as an intermediate host to a number of parasites (Torchin et al. 2001) and may function as a source of infection by transmitting pathogens to predators including birds, fish and mammals (Bush et al. 1993; Lotz et al. 1995).

It is accepted that predator–prey interactions might affect disease transmission and alter different trophic levels in an ecosystem (Marcogliese 1995) and even affect pathogen persistence in the host species (Hall et al. 2005). It is recognized that predator inhibition or enhancement of the pathogen is ecosystem specific and needs to be explored independently for each specific situation (Moore et al. 2010).

Different routes of entry for diseases and pathogens seem to be possible for *C. maenas.* Firstly, due to ingestion of disease infected tissue (www[1]) *C. maenas* is a mobile predator feeding upon Pacific oysters (McManus 1988) and preferentially targeting moribund (and thus potentially infected) individuals compared to healthy individuals (Moore 2002), resulting in direct take up of pathogens or diseases. Secondly, disease intake could happen by intraspecific contact of diseased scavengers and also cannibalism (Moksnes et al. 1998; Moksnes 2004). In addition, during respiration the gill tissue of *C. maenas* is in direct contact with infected particles in the water column, and the gills of *C. maenas* are

recognized as a selective interface between the external environment and the internal milieu (www[1]; Henry et al. 2012).

Differences in crab morphology, like coloration, sexual and life stage migrations are associated with ecosystem characteristics (Stevens et al. 2014). Within the intertidal zones shore crabs are well known to be migrants, both on a tidal and seasonal basis (Crothers 1968) with specific migratory behavior for different size classes and molt stages (Hunter & Naylor 1993). It is not well known how man-made structures, like oyster trestles and a virus infected culture species, might influence the natural migration patterns and behavior of *C. maenas*.

In this study, disease dynamics involving OsHV-1 µVar, *C. gigas* and a mobile scavenger, *C. maenas* was studied at two Irish Pacific oyster culture sites, responsible for the majority of production of Irish *C. gigas* with a history of OsHV-1 µVar and having different ecosystem characteristics. The role of *C. maenas* as a potential carrier, reservoir or alternative host of OsHV-1 µVar was investigated, taking into consideration the potential extension range of the virus in crabs as they migrated up and down the intertidal zone, associated with changing morphological and ecological characteristics during the crab's life cycle. The nature of the role of crabs in viral transmission was determined by laboratory-based trials. The focus of the study was to gain a better understanding of how the virus might sustain itself in the marine environment once introduced into a particular habitat and give a better insight into the potential wider ecosystem impacts of such introductions.

2.2. Material and methods

(1) Field trial

Study sites

Invertebrate sampling took place at two main Irish oyster culture sites, with different habitat structure; Dungarvan, Co. Waterford (52.0936 °N -7.6204°W) and Carlingford Lough, Co. Louth (54.0733°N -6.1994°W), approximately 245 km apart (Figure 1). Both sites are the main areas of production of Irish *C. gigas* and have a history of OsHV-1 µVar and oyster trestles are held in intertidal area with a tidal cycle of approximately 7-9 hours of emersion depending on neap or spring tides (Oyster farmers Pers. Comm.).



Figure 1. Crassostrea gigas culture site at Dungarvan, Co. Waterford and Carlingford Lough, Co. Louth, Ireland.

The oyster culture site in Dungarvan is sheltered, being almost closed off by the linear Cunnigar spit to the east (www[2]). Intertidal habitats are dominated by sandflats and it has mudflats at the edge of saltmarsh habitats. The water quality of Dungarvan Harbour varies from moderate to good, representing unpolluted water and acceptable levels of biochemical oxygen demand (EPA 2015). The oyster culture site in Carlingford Lough has a gravelly substrate covered by 3-5cm of muddy silt. Carlingford Lough, fed by the Newry River, has generally shallow waters of 2-5 m. Water quality within the lough is good; mean salinity is 32.5 and the annual temperature varies between 3 - 20°C (www[3]).

Environmental (salinity, pH, temperature) data loggers (Star-Oddi) *in situ* at the oyster trestles were used to measure and record water temperature continuously every hour from the end of May until the end of August 2015 at both sites, however, due to a technical issue with the logger, data was not recorded from the end of June to the end of July at Dungarvan. Average water temperatures were calculated as average temperature per day for the time submerged.

Macroinvertebrate sampling

Up to 30 crabs were collected randomly on the mid to low shore at the oyster trestles and at the high shore approximately 500 m from the trestles, every two weeks from the end of April until the end of August 2015 to detect possible infection of the virus. At Dungarvan, *C. maenas* were sampled directly from the oyster bags on the trestles approximately 1 foot above the sediment, as no crabs were observed outside the oyster bags. At Carlingford Lough, crabs were sampled outside the oyster bags on the sediment around the trestles. At the high shore at both sites, *C. maenas* were sampled from rock pools and rocky outcrops. In addition, to detect baseline levels of virus in the natural host, at every sampling date, 30 *C. gigas*, originally imported from French hatcheries which were selectively bred for resistance to the virus (Oyster farmers Pers. Comm.), were collected at the oyster trestles at both sites.

In total, 806 crabs and 510 oysters were collected. Dungarvan was sampled nine times, with 60 crabs sampled at the high shore (as it was difficult to find crabs at this location) and 270 crabs and 270 oysters at the trestles. Carlingford Lough was sampled eight times with 238 crabs sampled at the high shore and 238 crabs and 240 oysters at the trestles.

Morphometric characteristics of C. maenas

Weight (g) and carapace width (mm) were recorded using a balance scales and vernier calipers. Carapace width was divided into 4 different size classes, Class 1: 9.3-20 mm, Class 2: 20.1-30 mm, Class 3: 30.1-40 mm, Class 4 > 40.1 mm. Weights were divided into 4 different weight classes, Class 1: 0 – 10.0 g, Class 2: 10.1 – 20 g, Class 3: 20.1 - 30 g, Class 4 >30.1 g. Classification of crab carapace colour (brown, green and red)/moult stage and sex was noted by gross visual examination.

(2) Laboratory transmission trial of OsHV-1µvar from Carcinus maenas to Crassostrea gigas

A laboratory transmission trial was designed to determine the nature of positive results detected in the wild and to assess the possibility of viral transmission from

the crabs to oysters. Naïve *C. gigas* (n=180) with an average weight of 3.4 g and an average length of 31.9 mm, which had never been exposed to OsHV-1 µVar and proven to be naïve by the Marine Institute (www[4]), were obtained from a hatchery at New Quay, Galway Bay (53° 09′ 16·27″ N, 9°04′ 58·19″ W). Crabs with an average weight of 18.5 g and an average carapace width of 40.2 mm were randomly collected from Carlingford Lough in September 2015 where OsHV-1 µVar had been detected in oysters and in crabs during the field study. Prior to the start of the trial, 30 naïve *C. gigas* and 30 C. *maenas* were screened for OsHV-1 µVar by polymerase chain reaction (PCR), to confirm the oysters were uninfected and to determine if the virus could be detected in *C. maenas*. Before placing in tanks, *C. maenas* were washed several times in ddH_2O to remove any pathogens that may have been incidentally attached to their external body/shell. 10 l tanks were filled with 8 l of UV treated seawater. In Ireland, water temperatures often remain below the threshold temperature of 16°C (www[5]) and to imitate natural water temperatures, a lower temperature was chosen during the laboratory trial. UV filtered natural seawater and animals were held at 14°C in a constant temperature (CT) room with a salinity of 35 ppt. At the start of the trial a water conditioner (1 ml of Aqueon) was used, to keep the water quality to an optimum. The experimental set up consisted of two control tanks each containing 30 naïve oysters and three experimental tanks, which contained 30 naïve oysters and 10 virus-exposed crabs each. The trial ran for 14 days. The tanks were checked twice a day for mortality (open shells) and dead individuals were removed and screened for OsHV-1 µVar if tissue was present and of a suitable quality, but no tissues could be recovered for screening from these animals due to predation. After day 2 (48 hours), Day 4 (96 hours), Day 7 (168 hours) and Day 11 (264 hours), living oysters (n=3) were arbitrarily selected from the tanks each time to screen for OsHV-1 µVar. All individuals, oysters and crabs, still alive at the end of the experiment were removed and screened for OsHV-1 µVar.

(3) Molecular diagnostic screening

DNA extraction

Gill and internal tissues made up of connective, digestive and reproductive tissues of both oysters and crabs were stored in 70% ethanol for DNA extraction. Prior to extraction, tissues were washed in double deionized water (ddH_2O) thoroughly

and blot dried using tissue paper. DNA extraction was performed using the Chelex-100 methodology. Tissue samples from the invertebrates (approx. $5mm^2$) were placed in a 10% chelex solution (100 microlitres volume) (Sigma Aldrich) and following the samples were placed in a thermo Hybaid thermal cycler for 1 hour and 10 minutes heated at 99°C to facilitate cell lysis (Walsh et al.1991). To avoid false negatives, a subsample of DNA samples (n=30) were checked for DNA quantity and quality by using a NanoDrop 1000 spectrophotometer following protocol T042-TECHNICAL BULLETIN NanoDrop Spectrophotometers (www^6). From the samples collected from Dungarvan during the field trial, DNA was extracted from 330 individual *C. maenas* with 330 gill and 330 internal tissues being screened from those crabs and 270 *C. gigas* were sampled with 270 gill tissues being screened. DNA was extracted from 476 *C. maenas* (476 gill and 476 internal tissues screened) and 240 *C. gigas* (240 gill tissues screened) in Carlingford Lough. For the laboratory trial, DNA was extracted from 58 *C. maenas (*58 gill and 58 internal tissues screened) and for 137 C. *gigas* (137 gill tissues screened).

Polymerase chain reaction (PCR)

Standard PCR to detect OsHV-1 and variants was performed following the protocol of Lynch et al. (2013) by using OHVA/OHVB primers. All PCRs used a total of 2 μL genomic DNA template per individual. Expected size of amplified PCR products for OsHV-1 μVar was 385 bp and PCR was carried out in 25 μL containing 12·9 μL ddH_20, 5 μL, 5× buffer, 5 μL dNTPs (0·2 mM), 0·5 μL $MgCl_2$ (25 mM stock), 0·25 μL of each primer (100 pmol mL^{-1} stock) and 0·1 μL Taq DNA polymerase. Positive controls (duplicate) consisting of OsHV-1 μVar infected oyster tissue and negative controls (duplicate) of double distilled water (ddH_2O) were used for each PCR. Thermo cycling conditions were performed by initial denaturation of 1 min of 95 °C, following by 35 cycles including a denaturation step of 20 seconds at 94 °C, an annealing step of 30 seconds at 56 °C and an elongation step at 72 °C and finishing with a final elongation step of 7 minutes at 72 °C by using a thermo Hybaid PCR express thermal cycler (Lynch et al. 2013). Presence of amplified PCR products was confirmed by electrophoresis using a 2% agarose gel stained with ethidium bromide (10mg/l stock) and was run

with an electrical charge of 110V for 45-60 minutes.

Quantitative polymerase chain reaction (qPCR)

Quantitative PCR (qPCR) was carried out to determine the viral load of samples deemed positive for OsHV-1 µVar by PCR, on a subsample of *C. maenas* collected in the field trial (n = 43) and *C. maenas* (n = 24) and *C. gigas* (n = 5) in the laboratory trial, following the protocol "http://www.eurl-mollusc.eu/content/download/42545/578238/file/OsHV-" (www[7]) using primers HVDP-F and HVDP-R (Webb et al. 2007). All qPCRs used a total of 5 µL genomic DNA template per individual (duplicate). The qPCR mix was carried out in 25 µL containing 12.5 µl 2 x Brilliant Sybr Green ® Q PCR Master Mix, 2.5 µl HVDP-F (5µM) and 2.5 µl HVDP-R (µM) primers and 2.5 µl ddH_2O. Standards (plasmids) were used to detect the exact amount of viral copies μl^{-1} of genomic DNA in tested samples. Standard curves were prepared by diluting a viral DNA suspension of 10^8 viral copies μl^{-1} of genomic DNA of OsHV-1. Q PCR plates included 5 dilutions of 10^5, 10^4, 10^3, 10^2 and 10^1 viral copies μl^{-1} of genomic DNA. Negative controls (duplicate) of double dionized water (ddH_2O) were used for each qPCR. Thermo cycling conditions were performed by initial denaturation of 2 min of 50 °C and 10 min at 95°C, following by 40 cycles of 15 seconds at 95°C and 1 min at 60 °C and a melt curve of 95°C for 15 seconds, 60 °C for 1 minute, 95°C for 30 seconds and 60 °C for 15 seconds by using a thermo Hybaid PCR express thermal cycler (www[7]).

In situ hybridization (ISH) with DIG labelled probe

In situ hybridization (ISH) was carried out to detect the viral genome within different tissue sections of virus infected individuals. For each individual collected in this study, a section of internal tissue including gills, digestive and reproductive organs, were removed for histological analysis and immediately fixed in Davidson's solution at 4 °C for 24-48 h after which they were placed in 70% ethanol. *In situ* hybridization assays were carried out on *C. gigas* and *C. maenas* from the field trial screened negative (n=3 per species) and positive (n=3 per species) for OsHV-1 µVar by PCR. Samples were processed (Shandon Citadel 1000) and sectioned to 7 µm tissue thickness. ISH was carried out using a digoxigenin (DIG)-labelled (OsHV-1 µVar specific) probe (Lynch et al. 2010).

Sections were viewed and viral cells were noted with a Nikon Eclipse 80i and images were captured using NIS elements software (at 100×, 200× and 400×).

Direct Sequencing

Direct Sanger sequencing of DNA of PCR products (385-bp) amplified in *C. maenas* from the field trial (n = 3 for gill tissue and n = 3 for internal tissue) was carried out to confirm OsHV-1 µVar detection. DNA was isolated from PCR products of separate tissues (pooled 4 replicates per tissue to increase the DNA concentration). Qiagen Qiaquick gel extraction kit was used to isolate and clean up the DNA, prior to direct sequencing of both forward and reverse strands of DNA by Eurofins MWG. Sequences were matched by BLASTn nucleotide database (https://blast.ncbi. nlm.nih.gov/) to confirm true infection of OsHV-1 µVar.

(4) Statistical analyses

Statistical analyses were performed in Statistical model program R studio (R core team 2013). Normality was tested using the Shapiro-Wilks Normality test. A Mann Whitney test was used to determine if there was a significant difference between the mean weight and mean carapace width between the sites. Pearsons Chi-squared tests were used to compare sex and colour between sites and within sites at the two shore heights and to test for differences in prevalence of OsHV-1 µVar within gill and internal tissue for crab length classes, weight classes, sex and colour/moult stage. For all analyses, a critical value of 0.05 was used to confirm significant results. Data are presented as mean ± standard error.

2.3. Results

(1) Field trial

Prevalence of OsHV-1 µVar in Crassostrea gigas

Herpes virus was detected in oysters at both sites during the study period. Overall prevalence of OsHV-1 µVar detected by PCR in oysters at the two sites for the duration of the field trial was low with a mean prevalence of 3.75% at both sites, with a range in prevalence of 0-27% at Dungarvan and a range of 0-23% at Carlingford Lough. However, the mean temperature over the study period was 15.0°C for Dungarvan and 14.2 °C for Carlingford Lough, with the overall water

temperature during summer 2015 being low, rarely reaching temperatures of 16 °C or higher (Figure 2). At the farms, a tidal cycle of approximately 7-9 hours of emersion depending on neap or spring tides are common (Oyster farmers, pers comm) and therefore the sites were exposed to higher temperatures during low tides. Periods of air temperature above 16 °C were measured from the end of May (www[8]). Significant difference in prevalence between months were observed for both Dungarvan (X^2= 25.15, df = 4, P < 0.01) and Carlingford Lough (X^2= 17.95, df = 4, P < 0.01) with highest prevalence in June for both sites.

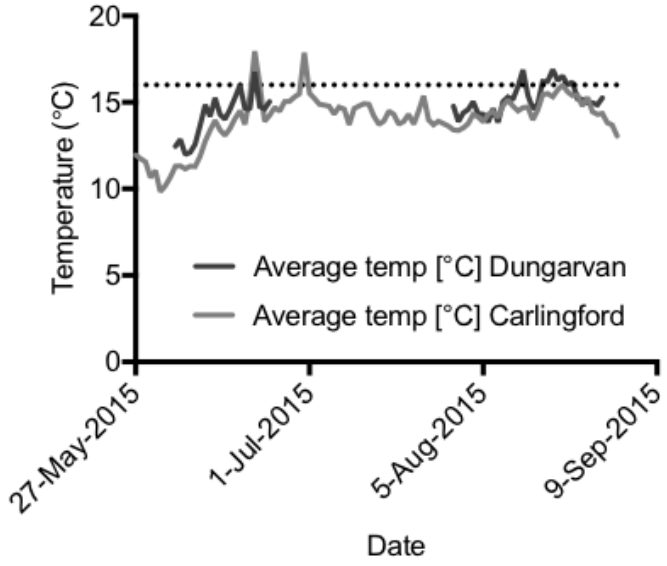


Figure 2: Average water temperature for Dungarvan and Carlingford Lough (Dotted line representing the threshold temperature of virus activation at 16 °C)

Crab morphometrics

C. maenas were significantly larger (Mann-Whitney, P < 0.01) and heavier (Mann-Whitney, P < 0.01) at Carlingford compared with Dungarvan (Table 1). All four carapace classes were present at both locations and shore heights, with crabs at Carlingford Lough having significantly larger carapace widths compared to crabs at Dungarvan (X^2=131.52, df =3, P < 0.01). Within Dugarvan, larger crabs were significantly more abundant at the trestles (X^2=32.12, df =3, P < 0.01), while no significant difference in carapace widths was observed between crabs at the trestles and high shore in Carlingford Lough (X^2=1.71, df =3, P > 0.05). All four crab weight classes were present in Carlingford lough while three weight classes were observed at Dungarvan, no significant differences were found for weight

classes between high shore and trestles. A significantly higher (X^2=10.66, df=1, P < 0.01) female-male ratio of 1:0.6 in Carlingford Lough was observed relative to 1:1 in Dungarvan. Within each site, no significant difference in female-male ratio was observed between the high shore and trestles. Green, brown and red coloured *C. maenas* were observed at Dungarvan and Carlingford Lough. At Dungarvan, green, recently moulted crabs were most abundant, followed by brown and red (1:4.1:2.2 for red:green:brown crabs), while at Carlingford Lough brown crabs were most common, followed by green and red crabs (1:1.9:2.0 for red:green:brown crabs). Colour ratio did differ significantly between both culture sites (X^2=23.085, df =2, P < 0.01). Within sites only a significant difference within coloration was observed between high shore and trestle at Carlingford Lough (X^2=14.73, df=2, P < 0.01), with significantly more green crabs at the trestles and brown crabs at the high shore.

Table 1. Weight and carapace width data for Carcinus maenas at the high shore and at oyster trestles in Dungarvan and Carlingford Lough.

	Average weight (gram)	Weight range (gram)	Average carapace width (mm)	Carapace width range (mm)
Dungarvan	4.2 ± 0.2	0.21 - 26.4	24.8 ± 0.4	9.8 - 50.5
High Shore	2.2 ± 0.4	0.21 - 23.7	20.1 ± 0.9	9.8 - 50.5
Trestle	4.6 ± 0.2	0.34 - 26.4	25.8 ± 0.4	11.9 - 49.4
Carlingford Lough	11.2 ± 0.5	0.23 - 52.0	33.5 ± 0.5	9.4 - 64.1
High Shore	11.3 ± 0.7	0.29 - 52.0	33.7 ± 0.7	10.5 - 64.1
Trestle	11.2 ± 0.7	0.23 - 47.5	33.4 ± 0.7	9.4 - 63.2

Viral detection in C. maenas

OsHV-1 µVar was detected in *C. maenas* during the entire five-month field trial at both culture sites (Figure 3).

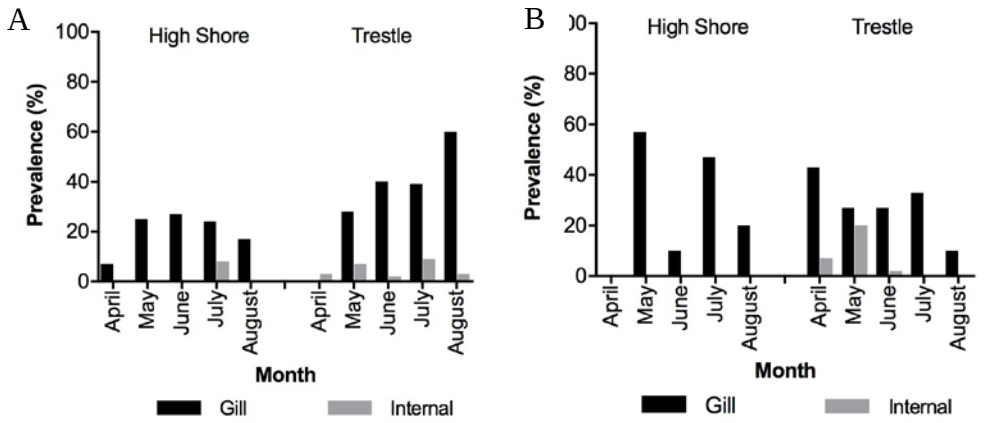


Figure 3: Prevalence of OsHV-1 µVar in Dungarvan (A) and Carlingford Lough (B) for gill and internal tissues of C. maenas at high shore and trestles per month

The mean prevalence of infection in both tissues of *C. maenas* was higher at Dungarvan at 18.3% (n = 121/660) compared to Carlingford Lough with 16.3% (n = 155/952), but not significantly different (X^2= 1.73, df =1, P > 0.05). The overall prevalence of OsHV-1 µVar in the screened tissues of *C. maenas* for both sites combined was 17.1% (n = 276/1612). For those infected tissues, the virus was detected only in gill tissue in 89.9% (n = 241/268) of the crabs, in only the internal tissue of the crabs in 7.1% (n = 19/268) of animals and was observed in both gill and internal tissues in 3.0% (n = 8/276) of crabs. This pattern was present at both shore heights in Dungarvan and Carlingford Lough (Table 2).

Table 2. Prevalence of OsHV-1 µVar by PCR in Carcinus maenas gill and internal tissues at the oyster trestles and high shore at Dungarvan and Carlingford Lough.

	Trestle		High Shore	
	Prevalence gill	Prevalence internal	Prevalence gill	Prevalence internal
Dungarvan	35.6% (n=96/270)	5.6% (n=15/270)	21.6% (n=13/60)	1.7% (n=1/60)
Carlingford Lough	27.7% (n=66/238)	6.3% (n=15/238)	29.4% (n=70/238)	0% (n=0/238)

qPCR analyses indicated different viral loads for a subsample (n = 43) of the crabs' gill tissue and internal tissue, screened positive initially with PCR. Overall the viral load was low, with up to 100 viral copies μl^{-1} of genomic DNA in most crabs (n = 36) screened by qPCR, while some individuals (n = 7) had higher viral DNA load, with the highest load detected being $>10^4$ viral copies μl^{-1} of genomic DNA (Table 3).

Table 3. Mean viral copies μl^{-1} of genomic DNA in samples of Carcinus maenas collected from the culture sites and deemed positive for OsHV-1 µVar by PCR - =no samples screened.

	$< 10^2$	**10^2-10^4**	**$>10^4$**
Gill tissue			
Dungarvan High Shore	100% (n=2)	0%	0%
Dungarvan Trestle	94.7% (n=18)	5.3% (n=1)	0%
Carlingford Lough High Shore	66.6% (n=4)	33.3% (n=2)	0%
Carlingford Lough Trestle	75.0% (n=6)	25.0% (n=2)	0%
Internal tissue			
Dungarvan High Shore	-	-	-
Dungarvan Trestle	66.6%(n=4)	16.7% (n=1)	16.7% (n=1)
Carlingford Lough High Shore	-	-	-
Carlingford Lough Trestle	100% (n=2)	0%	0%

One forward and one reverse DNA sequence was generated from one sample of *C. maenas* in the Direct sequencing. After sequencing of the PCR products, BLASTn analysis showed a match with an average of 96% (94-98%) similarity and 99% identity with OsHV-1 µVar (KU861511.1) for the sequence of the PCR-amplified products for *C. maenas*. *In situ* hybridization staining of crab (digestive and connective internal tissues) and oyster tissue sections resulted in a positive signal for OsHV-1 µVar in PCR-positive crabs (Figure 4A, 4B, 4C) and oysters, while PCR-negative crabs (Figure 4D) and oysters indicated no staining (i.e. no infection) in any tissue.

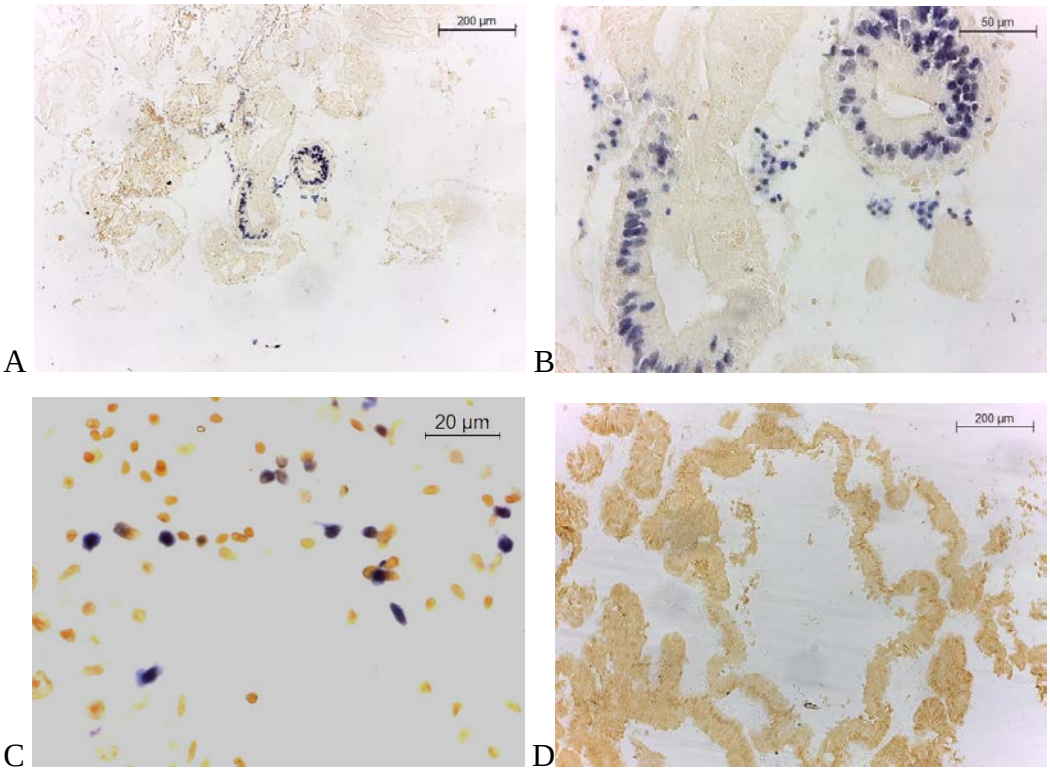


Figure 4: ISH staining of OsHV-1 µVar infected blood cells (dark blue) in connective tissue (Digestive Tract) of C. maenas naturally exposed to an OsHV-1 µVar endemic area (A + B +C) and uninfected tissue (D).

No temporal pattern was observed for OsHV-1 µVar prevalence in both tissue groups, however prevalence in gill tissue was significantly lower in April compared with all other sampling months (May, June, July and August (A Chi-Post hoc with a p adjust: Benjamini & Hochberg, $P < 0.05$) for both sites. Patterns in prevalence between sites at high shore and lower shore for different tissue groups at Carlingford and Dungarvan only showed significantly higher prevalence of OsHV-1 µVar in the internal tissues of crabs at the trestles ($X^2=13.50$, df=1, $P < 0.01$) compared to higher shore.

OsHV-1 µVar was detected in all length and weight classes sampled at both sites and shore heights. No clear trend was found for the prevalence of OsHV-1 µVar in the crab gill and internal tissues for the different length classes and weight classes. No significant difference was observed for different carapace width classes, different weight classes and crab tissue screened. Females showed a higher prevalence in gill (33.7%) and internal (4.9%) tissues compared with males (gill (28.4%) and internal of (2.6%)) however these results were not significant (gill;

X^2=3.55, df =1, P > 0.05 and internal; X^2=2.120, df =1, P > 0.05). Significant differences in the colouration/moult stage of *C. maenas* and the prevalence of OsHV-1 µVar were found for gill tissue detection (X^2=10.20, df=2, P < 0.05) with the highest prevalence being observed in recently moulted green crabs (37.5%), followed by brown crabs (30%) and red crabs (26.6%), however for internal tissue no significant differences were observed for the different coloured individuals and OsHV-1 µVar prevalence (X^2=0.21, df =2, P > 0.05). This pattern was mainly observed at the trestles and not at the high shore.

(2) Laboratory transmission trial of OsHV-1 µVar from Carcinus maenas to Crassostrea gigas

In the initial sample screening, oysters were uninfected with OsHV-1 µVar as expected, while *C. maenas* (only gill tissues) showed a low prevalence of OsHV-1 µVar (<10%) (Table 4), with an average of 11 viral copies μl^{-1} of genomic DNA.

Table 4. Prevalence of OsHV-1 µVar in Crassostrea gigas and Carcinus maenas by PCR in the initial sample and experimental sample of laboratory transmission trial.

	Prevalence gill by PCR	Prevalence internal by PCR
Initial *C. gigas*	0% (n=0/30)	-
Initial *C. maenas*	10% (n=3/30)	0% (n=0/30)
Experimental *C. gigas*	6.5 % (n=5/77)	-
Experimental *C. maenas*	75.0% (n=21/28)	0 % (n=0/28)

All oysters in the two control tanks were still alive at the end of the trial. Oysters of one of the two control tanks (n = 30) were screened for prevalence of OsHV-1 µVar by PCR on the last day of the trial. All control individuals were negative for OsHV-1 µVar. In experimental tanks, total mortality observed in *C. gigas* was 14.4% (n=13/90 - n=8 in tank 1, n=1 in tank 2, n=4 in tank 3) exposed to *C.*

maenas, while *C. maenas* itself had very low mortalities with <10% (n = 2 out of 30 / n = 1 in tank 1, n = 1 in tank 2, n = 0 in tank 3). Cumulative mortality of *C. gigas* taking into account removal of 3 oysters per tank at day 2 (48 hours), Day 4 (96 hours), Day 7 (168 hours) and Day 11 (264 hours) (n = 36) was <25% (n = 13/54) (Figure 5). Despite daily screening of the tanks, open shells were counted and removed to assess mortality but the tissues in these shells were either too degraded for screening or had been removed by crab predation. As a result, infection levels in these 14 dead oysters could not be assessed and only alive *C. gigas* were screened. In addition, no tissue of the two dead crabs was left, possibly due to cannibalism. In the *C. gigas* experimental tanks, the first positive signal of OsHV-1 µVar occurred within 96 hours. After screening all experimental oysters, *C. gigas* showed an OsHV-1 µVar prevalence of 6.5% (n= 5/77) with up to 1.2 x 10^2 viral copies μl^{-1} of genomic DNA. The viral prevalence in *C. maenas* gill tissue was 75% (n = 21/28) with greater than 1.0 x 10^4 viral copies μl^{-1} of genomic DNA, no screened internal tissue showed infection (Table 4).

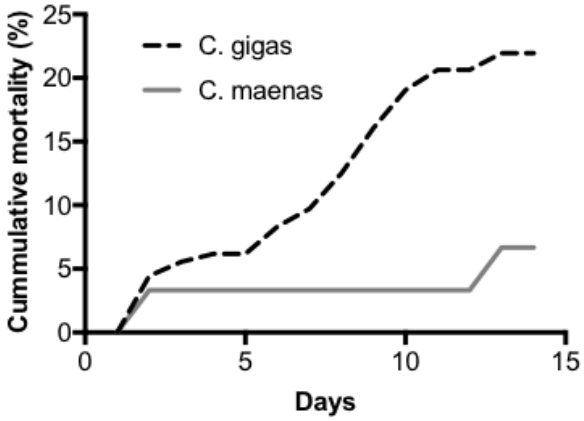


Figure 5: Overall cumulative mortality rates of experimental tanks with C. gigas and C. maenas (derived from grouping observations from 3 tanks)

2.4. Discussion

The study demonstrated that *C. maenas* can become infected with OsHV-1 µVar by using a range of protocols recommended by OIE including PCR, qPCR and *In Situ* hybridization (www.oie.int/fileadmin/Home/eng/Health_standards/aahm/current/chapitre_ostreid_herpesvirus_1.pdf). Although, we did not use the primer pairs as described in the OIE protocol, we were using primer pairs that we or colleagues have

successfully developed and have previously had published; PCR (Lynch et al. 2013) qPCR (Webb et al. 2007), ISH (Lynch et al. 2010).

This study indicates that the green shore crab *C. maenas*, an important mobile scavenger and predator in the intertidal area, can act as a carrier, reservoir and alternative host of oyster herpes virus, demonstrating that introduction of a virus through anthropogenic input, can have long-term and widespread ecosystem impacts, as the virus spreads amongst other cohabiting species. OsHV-1 µVar was detected in *C. maenas* at both culture sites and both shore heights, in all moult stages, crab sizes and in both crab sexes. While a seasonal effect could not be determined as the study concentrated on the summer months when viral impact is most pronounced, the virus was detected in *C. maenas* throughout the five-month study period. Highest prevalence of OsHV-1 µVar in the primary host, *C. gigas*, was detected in June at both sites. The low herpesvirus (<5%) prevalence observed in *C. gigas*, might be due to the unfavorable ambient temperatures with temperatures generally below 16 ºC during the study (Petton et al. 2013; Renault et al. 2014). Additionally, oysters selectively bred for resistance to the virus were used at the field trial in this study (Dégremont 2011) as this was what the farmers were culturing. As a scavenger, it is likely that *C. maenas* would preferentially target moribund (and thus potentially infected) *C. gigas* compared to healthy oysters (Moore 2002) and therefore possibly build up the virus while the abundance of infected *C. gigas* would decrease.

Although precautionary measures were taken in this study to wash and remove any incidental occurrence of OsHV-1 µVar on crab gill tissue, more detection of virus occurred in the gills compared to internal tissues, which suggests that the virus is not incidental on the gills and that the virus is being internalized in the tissue. In addition, ISH analyses in this study confirmed the positive detection OsHV-1 µVar internally in *C. maenas* digestive tissues, whereas Direct Sequencing confirmed OsHV-1 µVar within gill and connective *C. maenas* tissues. Higher prevalence in gills may indicate that crabs are being exposed via respiration rather than through feeding routes when initial exposure is occurring. With a widespread distribution of crabs around oyster trestles, with associated viral dispersion in the seawater (Schikorski et al. 2011), exposure in this way might be a likely first mode of uptake

for crabs. Moreover, lower internal infection of *C. maenas* might be the result of low infection of *C. gigas*, in this case the virus is not ingested by predation and scavening by crabs and less likely to migrate throughout internal tissues. The nature of the infection in crabs may differ to that observed in oysters with localization of the virus in crabs more likely in gills than dispersed throughout the connective tissues as observed in oysters.

While crab size and sex did not have any significant effect on the prevalence of the virus in the crabs, coloration/moult stage did, with green recently moulted crabs have higher levels of virus. This may suggest that this phase of the life cycle makes the animals more susceptible to infection, possibly due to easier access to tissues, or crabs being more immunocompromised during this phase.

The presence of the trestles, providing protection from predators, a readily available food supply in the form of diseased and dying oysters and acting as a nursery site for *C. maenas* replacing the high shore intertidal pools (Pers. Obs.), might result in abnormal behavior in *C. maenas*, which would have an impact on ecosystem dynamics. Previous studies observed *C. maenas* varying from 25 to 55 mm in carapace width in intertidal areas in the UK (Dare et al.1983), with smaller individuals found at high shore sites, and older *C. maenas* found lower down the shore (Hunter & Naylor 1993) and actively feeding upon *C. gigas* when they were present (Dare et al. 1983). Indeed, in Dungarvan, differences in size and weight of *C. maenas* was observed between shore heights, with larger and heavier individuals at lower shore (trestles). Also, in agreement with a previous natural behavioral study of *C. maenas* (Hunter & Naylor 1993), a significantly higher abundance of males was observed at the high shore in Dungarvan. Those normal behavioral and migration patterns were missing at Carlingford Lough, with juvenile *C. maenas* being observed in and around oyster trestles at high shore. It is important to note that at Carlingford Lough, random oyster bags were found at high shore and therefore highly likely to have altered normal behavioral and migration patterns of *C. maenas*. Other studies have noted the attraction of juvenile *C. maenas* to Pacific oyster trestles. A recent study that took place at Kingston Bay, Massachusetts (USA), an OsHV-1 µVar free site, where *C. maenas* is a non-native species, showed a significantly higher numbers of juvenile *C.*

maenas (1-15 mm CW) within mesh grow-out bags with oyster shells or living oysters compared to mesh grow-out bags without oyster shells at the high intertidal area (Lovely et al. 2015). *C. maenas* are known to moult all year around (Naylor 1962) and previous studies found green, brown and red coloured crabs at all sites and both shore heights (Lovely et al. 2015). This supports the findings of our study; all crab moult stages and corresponding carapace coloration were found during the sampling period at both shore heights.

In our transmission trial, first infection of OsHV-1 µVar in naïve *C. gigas* was detected after 4 days. Even though the temperature was held below the associated activation threshold temperature of 16 °C, a total prevalence of 6.5% OsHV-1 µVar was detected in *C. gigas* after 14 days. This suggests that the virus, at nonfavorable temperatures, could be maintained in the system by other marine species, like *C. maenas*, acting as a carrier and transmitting it to host species *C. gigas*. Transmission of OsHV-1 µVar to naïve *C. gigas* might have been a result of direct contact between *C. maenas* and *C. gigas* or through filtration of virus particles in the water or feaces excreted by *C. maenas*. The higher prevalence of OsHV-1 µVar in gills of experimental crabs (75%) after 14 days compared with the initial sample (10%) might be the result of reactivation of the virus due to stress of transport and artificial settings. *C. gigas* showed a cumulative mortality rate up to 25%, however it was not possible to screen dead *C. gigas* as though tanks were checked twice daily there was no tissue left in those dead animals. Therefore, it cannot be determined if *C. maenas* had predated on live animals or scavenged tissues when the oysters were moribund. Due to this, infection of *C. gigas* might have been underestimated as it could not be determined if those dead animals were infected or not. No virus was detected within internal tissues of *C. maenas*, suggesting that migration of virus from gills to internal tissues needs longer, only occurs through other transmission routes (e.g. ingestion) or that infection in the crab shows different patterns of viral presence in the tissues.

Abnormal mortalities of *C. gigas* have been associated with viral loads of OsHV-1 µVar higher than 10^4 DNA copies mg^{-1} (Schikorski et al. 2011; Pernet et al. 2012). These high viral loads were detected in a small percentage of living *C. maenas* in our experimental laboratory study, however mortalities in *C. maenas*

remained low (<10%). The transmission trial was performed under threshold temperature of 16 °C, to imitate natural summers in Ireland. Keeping in mind climate change, for future transmission experiments between crabs and oysters, it would be of interest to choose higher temperatures and investigate the difference in transmission dynamics**.** In addition, to gain better understanding of the viral dynamics between the species and migration of the virus within crabs it would be of interest to perform new experiments in the future by exposing highly infected oysters with naïve crabs.

The results of this study suggest that OsHV-1 µVar is highly adaptable and when the odds are in favour of the host i.e. when seawater temperatures are cooler and when disease resistant oysters are present, OsHV-1 µVar will sustain itself in the ecosystem outside the host species for a long period of time and can "species jump" to *C. maenas*. The pathogenicity of OsHV-1 µVar to *C. maenas* is not known and further studies would be required to elucidate the impact of the virus on *C. maenas* in the intertidal zone, however, due to *C. maenas's* mobility a greater geographic range extension of OsHV-1 µVar is likely. Our results suggest that man-made structures like oyster trestles might have an effect on the ecology of *C. maenas* facilitating the trophic transfer of OsHV-1 µVar within marine ecosystems, in particular, to cohabiting top predator species of crabs such as fish and bird species.

Authors Contributions

BB, SL and SC conceived the ideas and designed methodology; BB and AO collected the data; BB analyzed the data; BB led the writing of the manuscript with contributions and corrections from SL and SC. All authors contributed critically to the drafts and gave final approval for publication.

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Chapter 3.

Host plasticity supports spread of an aquaculture introduced virus to an ecosystem engineer

Abstract

The common cockle *Cerastoderma edule* is of commercial importance, but also plays an important ecological role in the marine ecosystem both as an ecosystem engineer and an important food source for protected bird species. However, *C. edule* is also a host to a large number of pathogens and diseases known to date on their macro- rather than microparasites. Cockle beds are found close to aquaculture and fisheries operations, which can be "hot spots" for infectious agents including viruses and bacteria. Ostreid herpesvirus-1 microVar (OsHV-1 µVar) has spread to many Pacific oyster *Crassostrea gigas* culture sites globally, where it has been associated with significant mortalities in this cultured bivalve. The impact of the virus on the wider ecosystem, is unknown. As the likelihood of released virus dispersing into the wider aquatic ecosystem is high, the potential impact on various trophic levels at culture sites could also be high, depending on the plasticity of the virus and the susceptibility of other intertidal species. As the cockle could have significant ecological impacts the was chosen to assess potential spread of the virus outside the oyster. In this study, OsHV-1 µVar presence and prevalence in wild cockles at culture sites, and the cockle's ability to transmit the virus to the oyster host at a low temperature of 15°C was investigated. The virus was detected in cockles, indicating that the cockles were acting as hosts for the virus. In subsequent laboratory-based trials, viral transmission was affected from cockles to naïve oysters, five days post exposure. The laboratory study also demonstrated the virus was active and transmitted at lower temperatures than previously though possible. The impact of the virus in cockle beds will need to be determined but this study demonstrates that this virus has the plasticity to infect bivalve species with a significant ecosystem role, potentially disrupting ecosystem function. In addition, it demonstrates the potential geographic range extension of the virus to uninfected areas via mobile predators of *C. edule*, including protected migratory bird and fish species.

Key words

Ostreid herpes virus-1 microVar, trophic, viral transmission dynamics, ecosystem engineer, species jump, pathogen-host-environment interplay, *Crassostrea gigas*, *Cerastoderma edule*

3.1. Introduction

Coastal and marine ecosystems are routinely used for fisheries and aquaculture purposes (Hattam et al. 2015; FAO 2017a; FAO 2017b). Aquauculture sites are located on intertidal mud and sandbanks, which have a diverse community of marine invertebrates, algae and vertebrates, including protected wading bird species (European Commission DG Environment, 2013), supporting a complex interwoven food web with a number of trophic levels. Pathogens and diseases are common in marine habitats (Lafferty et al. 2015), often as a result of a complex interplay between the natural host, its environment and the pathogen (Engering et al. 2013). This can have a significant impact on fisheries and aquaculture (Willman et al. 2009; Lafferty et al. 2015) due to associated intensive culture.

In Ireland, wild beds of *Cerastoderma edule (C. edule)* are often found at *Crassostrea gigas* (*C. gigas)* culture sites. *C. edule* is widely distributed in intertidal ecosystems in North Africa and Europe (Longshaw & Malham 2013; FAO 2017c) and is of commercial importance (Dare et al. 2004) particularly in the British Isles, The Netherlands, Spain and France (Carrasco et al. 2014; FAO 2017c). Moreover, *C. edule* plays a significant ecological role as an ecosystem engineer (Longshaw & Malham 2013; Morgan et al. 2013a; Donadi et al. 2015), by directly and/or indirectly altering the marine ecosystem. Using its physical structure it can directly shape its marine environment by forming reefs (Donadi et al. 2013a; Donadi et al. 2015) and as bioturbators it can mobilize sediments and affect sediment stability and accumulation (Montserrat et al. 2009; Donadi et al. 2013b; Donadi et al. 2015; Eriksson et al. 2017). *C. edule* functions as a link between primary producers (Eriksson et al. 2010) by being a suspension feeder and therefore indirectly changes levels of algae, nutrients/metals and sediments (Jones et al. 1994; Cesar & Frid 2009; Eriksson et al. 2017). It can also indirectly effect higher trophic levels (Lotze et al. 2006; Cesar 2009) by acting as an important food source for cohabiting marine species such as crustaceans, the shore crab *Carcinus maenas* (Beukema 1991) and brown shrimp *Crangon crangon*

(Beukema 1992), fish species European plaice *Pleuronectes platessa* (Pihl 1985) and protected wading bird species such as the Curlew *Numenius arquata* (Goss-Custard & Jones 1976) and the oystercatcher *Haematopus ostralegus* (Ponsero et al. 2009). Direct removal of ecosystem engineers like *C. edule* for sand extraction, by fisheries or a decrease in abundance due to mortality outbreaks can negatively affect overall species diversity of the ecosystem (Cesar 2009; Colen et al. 2013) and cause effects on water chemistry and sediment oxygen levels (Morgan et al. 2013b), which are ecosystem specific (McLaughlin et al. 2007; Cesar & Frid 2009). Ecosystem recovery by re-establishing species composition and trophic structure is often very slow after removing ecosystem engineers (Lotze 2005; Eriksson et al. 2010).

Cockle mortalities have been reported sporadically (Rybarczyk et al. 1996; Masski & Guillou 1999; Atkinson et al. 2003; Thieltges 2006; Beukema et al. 2010) caused by predation, unfavourable environmental circumstances, overfishing, but have also been often linked with pathogens and diseases (Ducrotoy et al. 1991). *C. edule* provides a habitat to a wide range of pathogens and diseases including viruses, bacteria, fungi (Microsporidia), Apicomplexa, Amoeba, Ciliophora, Perkinsozoa, Haplosporidia, Cercozoa, Turbellaria, Digenea, Cestoda, Nematoda, Crustacea and Nemertea (Longshaw & Malham 2013). Surfaced cockles are in contact with the water column and are more exposed to environmental stressors thus making them more likely to be susceptible to infection than their buried counterparts (Morgan et al. 2013a). Cockle mass mortalities have been observed with surfaced cockles (Blanchet et al. 2003; Morgan et al. 2013a), which are associated with poor health and are also seen as a “prelude to death” (Blanchet et al. 2003).

Herpesviruses, double-stranded DNA, seem to support a high plasticity (mutation rate) as large varieties of herpesvirus genotypes have been identified since their first detection such as Cyprinivirus in common carp *Cyprinus carpio* (Waltzek et al. 2005); Salmonivirus in salmon *Salmo salar* (Waltzek et al. 2009); and Ictalurivirus in white sturgeon *Acipenser transmontanus* (Kurobe et al. 2008). Herpesviruses and herpes-like viruses have been of interest as they have caused significant ecological and economic losses in wild and cultured marine

invertebrates during the past few decades (Hine et al. 1992; Comps & Cochennec 1993; Hine & Thorne 1997; Renault et al. 2001; Chang et al. 2005). One important global aquaculture bivalve species is the Pacific oyster *Crassostrea gigas* (FAO 2017d). *C. gigas* culture has been affected by significant mortalities, associated with ostreid herpesvirus-1 (OsHV-1) (Burge et al. 2007) and its variants including ostreid herpesvirus-1 microVar (OsHV-1 μVar) since the 1990's (Lynch et al. 2012; Prado-Alvarez et al. 2016). The environment can directly and/or indirectly affect the innate immune system of marine invertebrates (Galloway & Depledge 2001; Mydlarz et al. 2006). This makes traditional vaccination against infectious pathogens and diseases in these species impossible (Gestal et al. 2008) and therefore eradication of this pathogen is impossible in open waters (Garcia et al. 2011; Pernet et al. 2016).

Past studies have investigated the possibility of other marine invertebrates being carriers and or reservoirs of oyster herpes virus (OsHV) and ostreid herpesvirus-1 (OsHV-1), outside the known host *C. gigas,* and have detected oyster herpes virus in cohabiting marine invertebrate species such as the eastern oyster *Crassostrea virginica*, European flat oyster *Ostrea edulis* and Mediterranean mussel *Mytilus galloprovincialis* in America (Burge et al. 2011) and Chinese scallop *Chlamys farreri* (Ren et al. 2013). More recently ostreid herpesvirus-1 microVar (OsHV-1 μVar) was detected in different marine invertebrates including the Sydney cockle *Anadara trapezia* in Australia (Evans et al. 2017) and in cultured mussels *M. galloprovinciallis* in Italy (Domenneghetti et al. 2014) and in wild *Mytilus spp*. consisting of the blue *Mytilus edulis, M. galloprovinciallis* and hybrids of both parent species in Ireland (O'Reilly et al. 2017). Viral transmission between *Mytilus spp*. and Pacific oysters (O'Reilly et al. 2017) and *Carcinus maenas* and Pacific oysters (Bokelaar et al. 2018 – In Press / Chapter 2) was observed . However there remain still a lot of questions if those species function as carriers, reservoirs or novel host species.

Certain pathogens in marine ecosystems, are capable of mutations and as a result a selection of new variants occurs, which are described as "emerging infectious disease (EID)". EIDs are increasing in incidence, impact and geographic range (Jones et al. 2008) and can be divided into three groups: (1) the pathogen selected

a novel host, thus making a so called “species jump”, (2) the pathogen shows a novel trait within the same host or (3) geographic extension of the pathogen occurs (Engering et al. 2013). The driving factors behind those species jumps are still unclear, but ecological and, or, evolutionary drivers, such as a complex interaction between climate, farming practices and viral genetic re-assortment, have been assumed (Flanagan et al. 2011). As a result, new species selected by the pathogen might function as a source of infection, and act as carriers or reservoirs (Lynch et al. 2007; Lynch et al. 2010; Small & Pagenkopp 2011) or potential new hosts (Howard & Fletcher 2012; Engering et al. 2013; Schrauwen & Fouchier 2014). A carrier is defined as an incidental, asymptomatic host and a distributor of infection. A reservoir is not an incidential carrier of the pathogen, it can retain the pathogen permanently and transmit it back to the natural host (Haydon et al. 2002; Lynch et al. 2010). When pathogens select new species as alternative hosts, carriers or reservoirs, its geographic expansion and modifications in the entire ecosystem are often unpredictable (Engering et al. 2013).

There are examples that transfer of diseases through the trophic levels in marine ecosystems might modify interactions between host and non-host species, resulting in shifts in predator – prey interactions or top down and bottom up dynamics directly (Carpenter 1990) and/or indirectly. A direct shift was observed in the sea urchin *Diadema antilarum.* When sea urchins died after a disease outbreak, less grazing of kelp resulted in expansion of kelp forests and shifts in algae communities (Carpenter 1990). An indirect effect was observed for rainbow trout *Oncorhynchus mykiss* (Sato et al. 2012) and nematomorph parasites that infect crickets, a prey species of *O. mykiss.* When crickets became infected, rainbow trout consumed less crickets and consumed more benthic invertebrates, causing a shift in benthic invertebrate communities. Therefore, this predator-prey interaction indirectly resulted in a decrease in biomass of benthic algae (Sato et al. 2012). Besides changes in trophic levels, diseases and pathogens have proven to change the physical state of the ecosystem. This phenomenon is observed when trematodes infected the foot of the ecosystem engineer, the New Zealand cockle *Austrovenus stutchburyi.* When infected, the burrowing ability of the cockles reduced and it made predation by bird species easier. Birds dropped the empty shells after consumption, which created a new habitat for other marine species

(Mouritsten & Poulin 2010). Pathogens and diseases also affect biodiversity, energy flow, and abiotic characteristics of an ecosystem (Selakovic et al. 2014). A modelling study indicated that when *Daphnia dentifera* was infected with virulent yeast parasite *Metschnikowia bicuspidate,* fish species will prey more on *D. dentifera* resulting in decreased yeast levels (Duffy et al. 2012).

From an evolutionary perspective, plasticity of viruses is common and species jumps by viruses can occur easily, like Influenza A and Nipah virus, both viruses jumped between humans and other vertebrates (Engering et al. 2013). Those species jumps of viruses are also observed between aquatic species e.g. jumps of Novirhabdovirus between rainbow trout *Oncorhyncus mykiss*, pike *Esox lucius* and grayling *Thymallus thymallus* (Kahns et al. 2012). An example of a viral transmission in marine systems is viral hemorrhagic septicemia virus (VHS) which was detected for the first time in the wild in 1979 in Atlantic cod, *Gadhus morhua* (Vestergard & Olesen 1987). Since then it has been discovered in more than 80 fish species in both wild and cultured species (Sandlund et al. 2014), however, not all infected species and families show disease symptoms and therefore species might act as carriers rather than host species (Johansen et al. 2003). These findings suggest that the virus has the ability to adapt and select new reservoirs, carriers or possible novel host species. However, those viruses are mainly RNA or single stranded DNA (ssDNA) viruses (Inglis et al. 1979; Schütze et al. 1999; Harcourt et al. 2005). RNA or ssDNA viruses have normally very small genomes and will respond very rapidly to strong selective pressures and therefore are very likely to mutate (Domingo & Holland 1997; Holmes 2009). Due to spontaneous deamination, mutations are up to 100-fold greater in single stranded DNA compared with double stranded DNA (dsDNA) (Xia & Yuen 2005).

As the cockle plays a significant ecosystem role and the herpes virus and its variants to date have been observed in a range of species, this study investigated the potential of the virus to infect the cockle and the cockle's ability to act as a host, carrier or reservoir of infection. To assess what role (host, carrier or reservoir) the cockle might play in the persistence or invasion of the virus, a series of protocols and diagnostic methods were carried out (OIE 2017). The ultimate focus

of this study was to highlight the potential impacts of anthropogenic introduced pathogens in a wider ecological context.

3.2. Material and methods

The study was conducted in two parts – the first was a field study to screen cockles for the presence of the virus – the second part was a laboratory transmission trial to determine if *C. edule* from an ostreid herpesvirus-1 μVar endemic Pacific oyster culture site could transmit the virus to naïve *C. gigas*.

(1) Field trial

Field study sites

This study took place at the two main Irish oyster culture sites; Dungarvan, Co. Waterford (52.0936 °N -7.6204°W), which covers an area of approximately 25 km^2 (NPWS 2011) and Carlingford Lough, Co. Louth (54.0733°N -6.1994°W), which covers an area of approximately 50 km^2 (Ferreira et al. 2008; NPWS 2013) (Figure 1). For both culture sites OsHV-1 μVar has been present since 2009 (Morrissey et al. 2015).

In Ireland, oyster culture sites including Dungarvan and Carlingford Lough are known as Special Protection Areas (SPA) under the EU Birds and Habitats Directives as they support over 20,000 waterbirds during the non-breeding season and are of specific conservation interest and importance for bird species (NPWS 2011; NPWS 2013). Mobile predators like birds; oystercatchers *Haematopus ostralegus* and the common tern *Sterna hirundo* are observed in Irish coastal areas year round (www[1]) and are known to prey on *C. edule* 10-30 mm in shell length, on the upper shore (Reise 1985; Burdon et al. 2014).

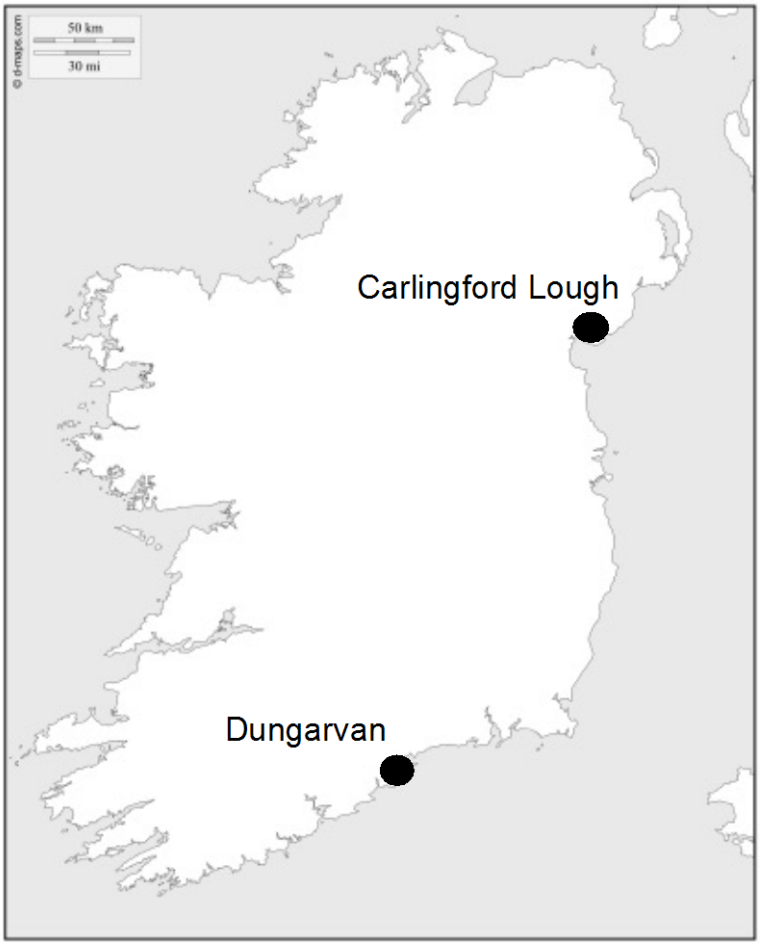


Figure 1. Crassostrea gigas culture site at Dungarvan, Co. Waterford and Carlingford Lough, Co. Louth, Ireland.

Environmental data

Environmental data loggers (Star-Oddi) supplied by Bord Iascaigh Mhara (BIM) were used to measure and record continuous water temperature values (every half hour) from the end of May until mid-August 2015 for both sites. Due to a technical issue, data was not recorded from the end of June to the end of July at Dungarvan.

Bivalve sampling

In this study, surfaced (gaping and non-gaping) cockles were randomly sampled at both *C. gigas* culture sites. Taking into consideration the potential extension range of the virus in the intertidal zone (e.g. by transport of infected particles through the water column), cockles were sampled around the oyster trestles (main culture site) during low tide and approximately 500-meter higher up shore. Thirty cockles were taken at the high shore at both sites and at the oyster trestles in Carlingford Lough (as no surfaced cockles were observed at the trestles in

Dungarvan). In addition, to detect baseline levels of virus in the natural host, at every sampling date, 30 *C. gigas*, originally imported from French hatcheries, were collected at the oyster trestles at both sites.

Cockles were collected nine times in Dungarvan (April 2015 to August 2015) and eight times in Carlingford Lough (April 2015 to August 2015). As cockles were not present at the trestles in Dungarvan, cockles (250 individuals in total) were only collected at the high shore. At Carlingford Lough, 445 cockles were collected for screening, 218 cockles at the high shore and 227 cockles at the trestles.

Cockles and oysters were processed for histology and molecular analysis on return to the laboratory (methods below ***(3)***).

(2) Laboratory transmission trial

Naïve *C. gigas* (n = 150, average weight of 3.0 g and average length of 31.3 mm), which had never been exposed to OsHV-1 µVar, were obtained from an Irish hatchery in October 2015. *C. edule,* (n = 93, average weight of 12.6 g and average shell length of 29.9 mm), were randomly collected in October 2015 from trestles in Carlingford Lough, where OsHV-1 µVar had been detected in oysters. Prior to the start of the trial, 30 naïve *C. gigas* and 30 *C. edule* were screened for OsHV-1 µVar by polymerase chain reaction (PCR), to confirm the oysters were uninfected and to determine if the virus could be detected in *C. edule*. Before placing in tanks, the exterior of the shells of *C. edule* were washed several times in double dionized water (ddH_2O) to remove any pathogens that may have been incidentally attached to their external shell. Four 10 l tanks were filled with 8 l UV filtered natural seawater and animals were held at 14°C in a constant temperature (CT) room with a salinity of 35. The experimental set up consisted of a control tank containing 30 naïve oysters and three replicate experimental tanks, which each contained 30 naïve *C. gigas* and 21 *C. edule*. As predation of crabs on oysters took place, the trial ran for 14 days. The tanks were checked several times daily for mortality and any gaping and/or dead individuals were removed to screen for OsHV-1 µVar by PCR. Oysters (n = 3) were sampled on Day 2 (48 hours), Day 4 (96 hours) and Day 7 (168 hours), to screen the naïve oysters for the virus to determine any replication of the virus was taking place by assessing viral loads. All individuals,

C. gigas and *C. edule*, still alive at the end of the experiment were removed and screened for OsHV-1 µVar. Since most cockles died within the first few days the trial was terminated at 14 days.

(3) Processing of individuals

Whole wet weight (g) and shell length (mm) of both field laboratory trial individuals were recorded using a balance scales and vernier calipers. In addition, shell rings were counted where number of shell rings presenting the age in years. Shell lengths were divided into five different length classes, Class 1: 0 – 8 mm; Class 2: 8.01-16 mm; Class 3: 16.01 – 24 mm; Class 4: 24.01 – 32 mm and Class 5: 32.01 – 40 mm. Weights were divided in 4 different weight classes, Class 1: 0 – 7 g; Class 2: 7.01-14 g; Class 3: 14.01 – 21 g and Class 4: 21.01-28 g. Correlation tests were performed between weight and age and between shell length and age to clarify if growth could be associated with life span. Age (represented by shell rings) were divided into different age classes, Class 1: 0 years, Class 2: 1 year; Class 2 years: 2; Class 3: 3 years; Class 5: 4 years; Class 6: 5 years and Class 7 > 5 years.

(4) Molecular diagnostic screening

DNA extraction

Gill and internal tissues (approx. 5mm^2) made up of connective, digestive and reproductive tissues of *C. edule* and gill tissue of *C. gigas* of both field and laboratory trial individuals were stored in 70% ethanol for DNA isolation to detect OsHV-1 µVar. Before DNA extraction took place, tissues were washed in double deionized water (ddH_2O) and blot dried using tissue paper. DNA extraction was performed using the Chelex-100 methodology (Walsh et al. 1991). Tissue samples were placed in a 10% chelex solution (100 microliters volume) (Sigma Aldrich) and following the samples were placed in a Hybaid thermal cycler for 1 hour and 10 minutes heated at 99°C to facilitate cell lysis (Walsh et al. 1991). A subsample (n = 30) of the cockle DNA was quantified by a spectrophotometer (NanoDrop 1000 spectrophotometer) following protocol T042-TECHNICAL BULLETIN NanoDrop Spectrophotometers to confirm DNA quantity and quality. Quantity of the DNA is represented as DNA per µg/l with a sufficient concentration of 40 µg/l. 260/280 and 260/230 ratios represent the quality of the

DNA. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~ 1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA. The 260/230 values for "pure" nucleic acid is often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants which absorb at 230 nm.

Polymerase chain reaction (PCR) OsHV-1 µVar

Standard PCR to detect OsHV-1 and variants was performed using the OHVA/OHVB primers (Lynch et al. 2013). Positive controls (duplicate) consisting of OsHV-1 µVar infected oyster tissue and negative controls (duplicate) of double distilled water (ddH_2O) were used in duplicate for each PCR. Agarose gel electrophoresis was carried out using a 2% agarose gel stained with ethidium bromide (10mg/l stock) and was run with an electrical charge of 110V for 45-60 minutes. Expected size of amplified PCR products for OsHV-1 µVar was 385 bp (Lynch et al. 2013).

Quantitative polymerase chain reaction (qPCR) OsHV-1 µVar

Quantitative PCR (qPCR) was carried out to determine the viral load of a subsample of cockles from the field trial (n = 76), including gill (n = 33) and internal tissue (n = 43) of cockles from both sites, deemed positive for OsHV-1 µVar by standard PCR. In addition, qPCR was performed on *C. edule* and *C. gigas* from the laboratory transmission trial, which were deemed positive for OsHV-1 µVar by standard PCR. qPCR conditions, master mix and thermo cycling conditions were carried out using the protocol "OsHV-1 detection and quantification by real Time Polymerase Chain Reaction using OsHV-1 DNA polymerase sequence"
(http://archimer.ifremer.fr/doc/00137/24814/22900.pdf). using primers HVDP-F and HVDP-R (Webb et al. 2007). All qPCRs used a total of 5 µL genomic DNA template per individual (duplicate). The qPCR mix was carried out in 25 µL containing 12.5 µl 2 x Brilliant Sybr Green ® Q PCR Master Mix, 2.5 µl HVDP-F (5µM) and 2.5 µl HVDP-R (µM) primers and 2.5 µl ddH_2O. Standards (plasmids) were used to detect the exact amount of viral copies μl^{-1} of genomic

DNA in tested samples. Standard curves were prepared by diluting a viral DNA suspension of 10^8 viral copies μl^{-1} of genomic DNA of OsHV-1. Q PCR plates included 5 dilutions of 10^5, 10^4, 10^3, 10^2 and 10^1 viral copies μl^{-1} of genomic DNA. Negative controls (duplicate) of double dionized water (ddH_2O) were used for each qPCR. Thermo cycling conditions were performed by initial denaturation of 2 min of 50 °C and 10 min at 95°C, following by 40 cycles of 15 seconds at 95°C and 1 min at 60 °C and a melt curve of 95°C for 15 seconds, 60 °C for 1 minute, 95°C for 30 seconds and 60 °C for 15 seconds by using a thermo Hybaid PCR express thermal cycler (www[7]).

In situ hybridization *(ISH)*

In situ hybridization assays (ISH) were carried out on cockles from the field survey that were negative (n = 3 for both sites) and positive (n = 3 for both sites) for OsHV-1 µVar when screened by PCR and qPCR. In addition, ISH was performed on infected (n = 3) and uninfected (n = 3) *C. gigas* from the laboratory trial. ISH was carried out using a digoxigenin (DIG)-labelled (OsHV-1 µVar specific) probe (Lynch et al. 2010).

Sequencing

Sequencing of genomic DNA from positive PCR products amplified from *C. edule* (n = 5) from the field screening (Carlingford n = 3 and Dungarvan n = 2) took place. PCR products in triplicate from each individual were pooled together to increase DNA concentration prior to being sent for direct sequencing to Eurofins MWG. DNA sequences that were generated by Eurofins MWG were matched against the National Center for Biotechnology Information (NCBI) nucleotide database with BLASTn to identify and confirm that the sequences were specifically OsHV-1 µVar strains.

(5) Statistical analyses

Statistical analyses were performed in statistical model program R studio [94] (R core team 2013). Normality was tested using the Shapiro-Wilks Normality test. Environmental data between sites were analysed by a student t-test. Morphometrics; length and weight between sites and shore heights were analysed by Mann Whitney U test. A Spearman rank order correlation was used to

determine if there was an association between shell rings and weight and between shell rings and length. Pearsons Chi-squared tests were used to compare length and weight classes and number of shell rings between sites and within sites at the two shore heights. Pearsons Chi-squared tests were used to test for differences of prevalence of OsHV-1 µVar for gill and internal tissue for cockle within sites at the two shore heights. For all analysis, a critical value of 0.05 was used to confirm significant results. Data are presented as mean ± standard error.

3.3. Results

(1) Field trial

Baseline levels of OsHV-1 µVar at Pacific oyster culture sites

The overall water temperature during summer 2015 remained low (< 16 °C). Overall prevalence of OsHV-1 µVar by PCR in *C. gigas* at Dungarvan (n = 270) and Carlingford Lough (n = 240) for the duration of the field trial was low (< 10%) with a mean prevalence of 6.0% and a range of 0-27% per month at Dungarvan and a mean prevalence of 6.0% with a range of 0-23% per month at Carlingford Lough (Figure 2).

Morphometrics Cerastoderma edule

Both age and weight (correlation coefficient = 0.85, $P < 0.01$) as well as age and length (correlation coefficient = 0.86, $P < 0.01$) indicated a strong positive correlation. Cockle weight, length (Mann Whitney U, $P < 0.01$) and age were significantly larger at Carlingford Lough compared to Dungarvan and within Carlingford Lough cockles were heavier, bigger (Mann Whitney U, $P < 0.01$) at the high shore, compared to cockles at the trestles (Table 1).

Table 1. Weight and shell height (± SE) data for C. edule at high shore and trestles in Dungarvan and Carlingford Lough

	Average weight (g)	Range weight (g)	Average shell height (mm)	Range shell height (mm)	Range shell ring classes
Dungarvan					
High Shore	4.6 ± 0.2	0.3 - 12.4	0.2 ± 0.3	9.3 - 28.4	1 - 5
Carlingford Lough					
High Shore	12.3 ± 0.3	4.8 - 22.0	29.3 ± 0.2	21.0 - 36.3	3 - 7
Trestle	9.9 ± 0.4	2.1 - 25.4	26.4 ± 0.4	14.9 - 36.3	2 - 7

Virus detection in Cerastoderma edule in the field

A subsample of cockle DNA (n = 30) was screened for DNA quantity and quality using a NanoDrop spectrophotometer. Regarding quantity all samples showed a sufficient amount of DNA higher than 40 μg/l and 80% of the samples had DNA higher than 100 μg/l. 260/280 ratio indicated values of 1.5-2.1. 260/230 ratios represented values ranging from 1.5-2.0, representing low contamination levels.

OsHV-1 μVar was detected in the cockles at both sample sites, and in both gill and internal tissues in the PCR screening**.** The overall prevalence of OsHV-1 μVar in *C. edule* for Dungarvan was 14.4% (14.4% at high shore) and in Carlingford Lough was 13.6% (12.8% at high shore and 14.3% at trestles). When both gills and internal tissues were screened, infection was detected in some animals just in the gills, in others just in the internal tissues and in some animals in both (Table 2). More individuals demonstrated the presence of the virus in the internal tissues (10.2 % n = 71/695) exclusively, followed by individuals with infection in gill only and individuals with infection in both tissues. This pattern however was only significantly different at the high shore at Carlingford Lough ($X^2 = 12.43$, df = 2, $P < 0.01$) and not at the high shore at Dungarvan ($X^2 = 2.29$, df=2, $P = 0.32$) and at the trestles at Carlingford Lough ($X^2 = 1$, df=2, $P = 0.61$) (Table 2).

Table 2. Prevalence of OsHV-1 µVar for gill and internal tissue of C. edule individuals at Dungarvan and Carlingford Lough

	Trestle			High Shore		
	Gill tissue	Internal Tissue	Both gill and internal tissue	Gill tissue	Internal tissue	Both gill and internal tissue
Dungarvan	-	-	-	6.4% (16/250)	9.6% (24/250)	6.4% (16/250)
Carlingford Lough	6.6 % (15/227)	8.8 % (20/227)	6.6 % (15/227)	5.0% (11/218)	12.3% (27/218)	4.1% (9/218)

Significant differences in prevalence between months were observed for both Dungarvan (X^2= 25.15, df = 4, P < 0.01) and Carlingford Lough (X^2= 17.948, df = 4, P < 0.01) with highest prevalence in May and lowest prevalence in April at both sites (Figure 2). No significant trends were observed for OsHV-1 prevalence and the size and age of the cockles (indicated by shell rings), with the virus being detected in all length and weight classes and within all ages.

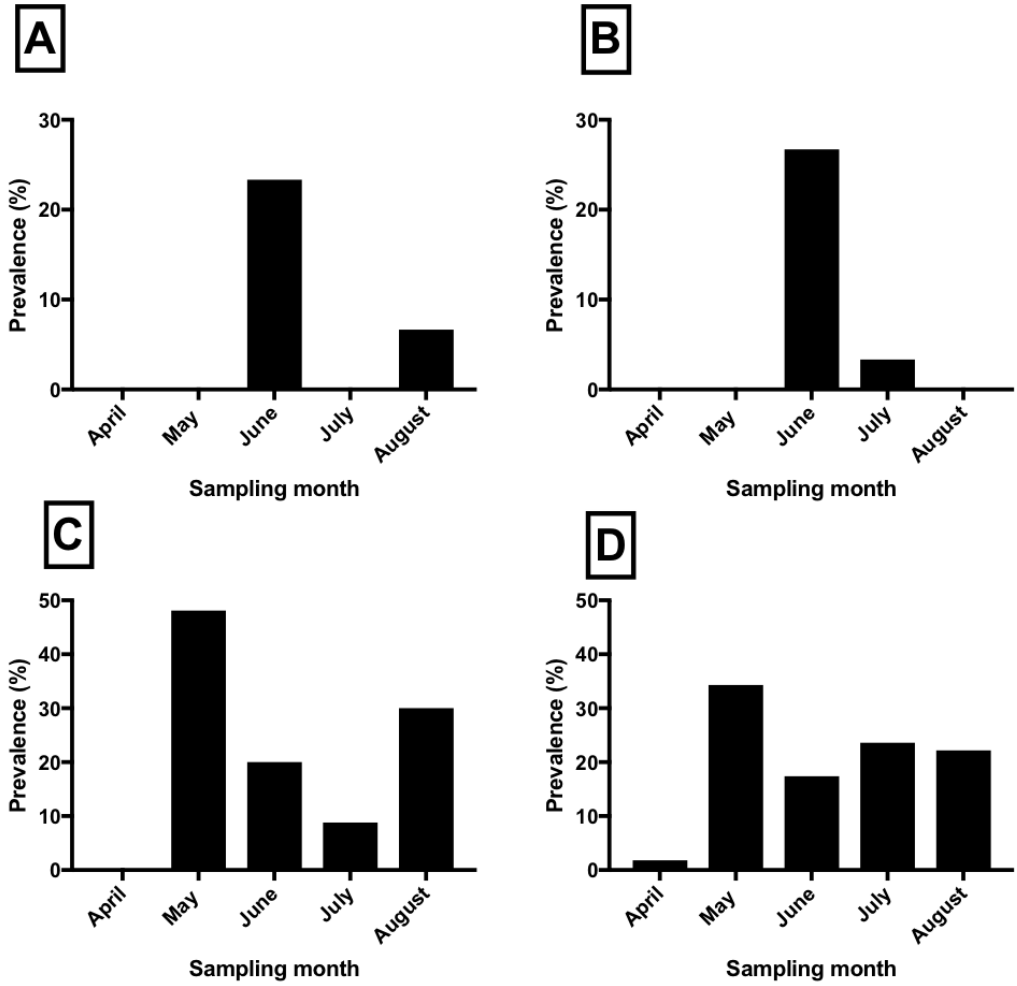


Figure 2. Overall prevalence (%) of OsHV-1 µVar for C. gigas at (A) Dungarvan and (B) Carlingford Lough and in C. edule at (C) Dungarvan and (D) Carlingford Lough over the study period April -August 2015

qPCR analyses indicated different viral loads for a subsample (including samples from both Carlingford Lough and Dungarvan) of cockle tissues (n = 76/695 is 10.9%) from the field trial (for more details Figure 3) these included gill tissue from 33 individuals and internal tissues from 43 cockles, all of which had a positive signal in the PCR for OsHV-1 µVar. Overall the viral load was relatively low with up to 10^2 viral copies/µl of genomic DNA in both gill tissue (81.8% n =27/33) and internal tissue (90.7% n = 39/43) screened by qPCR. A small number of gill tissues (6.1% n = 2/33) and internal tissues (7.0% n = 3/43) showed viral load varying between 10^2-10^4 viral copies/ µl of genomic DNA. High viral load

with >10^4 viral copies/ µl of genomic DNA was mainly observed in gill tissue (12.1% n = 4/33) compared with internal tissue (2.3% n = 1/43).

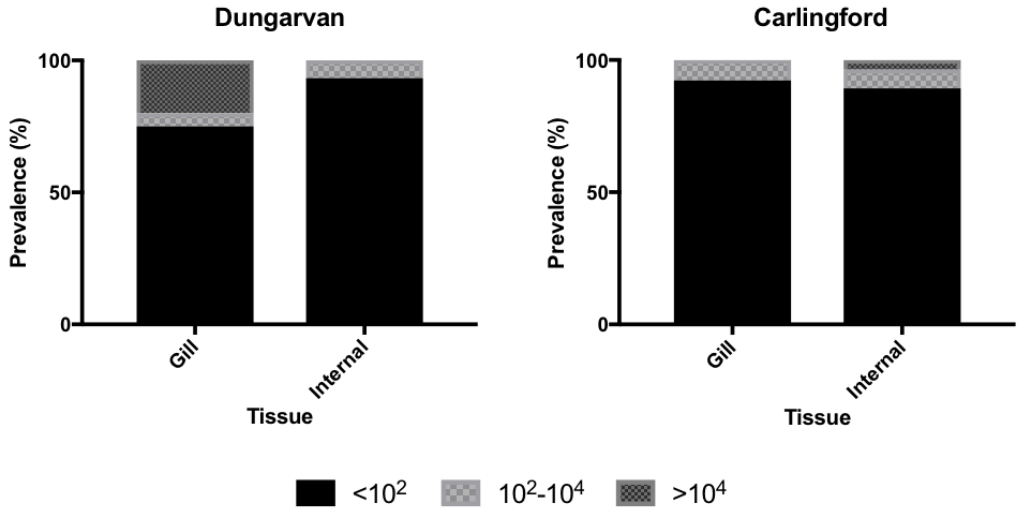


Figure 3. Mean viral loads in C. edule in percentage sampled from the culture sites and deemed to be positive for OsHV-1 microVar in the PCR

A positive signal for OsHV-1 µVar was observed visually by ISH for *C. edule* (83.3%, n = 5/6), collected in the field, in the digestive epithelia (Figure 4) from the laboratory trial, while PCR-negative *C. edule* and *C. gigas* indicated no infection in any tissue group. The quality of the tissue sections of *C. edule* were poor. High likely as they were all surfaced cockles, described as a prelude to death (Blanchet et al. 2003.

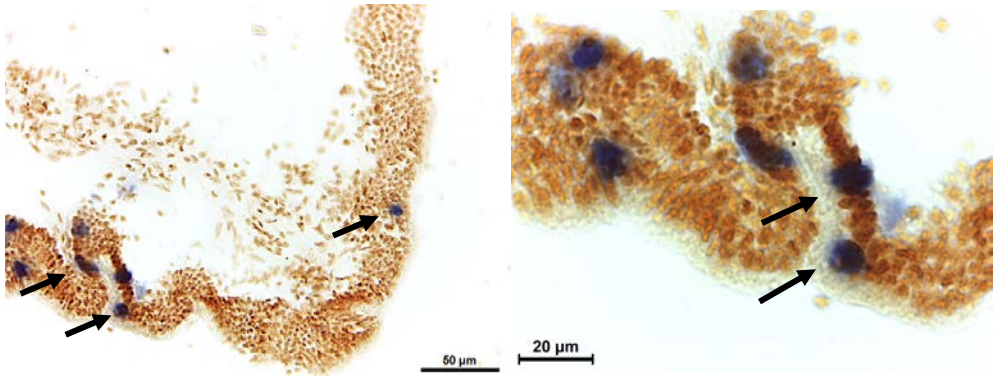


Figure 4. ISH staining of OsHV-1 µVar infected blood cells (dark blue) in digestive epithelia of C. edule

After sequencing of the PCR products of *C. edule* (n = 5), BLASTn analysis (www.ncbi.nlm.nih.gov/BLAST/) showed a match with OsHV-1 µVar for the sequence of the PCR-amplified products of *C. edule* with an average of 92.6% (88-95%) query coverage and 99% maximum identity to OsHV-1 µVar (Accession #KU861511.1).

(2) Laboratory transmission trial of OsHV-1 µVar

Prior to the trial commencing (collected in October 2015), 30 individuals of each species (cockles and oysters) were screened. Naïve *C. gigas* were uninfected with OsHV-1 µVar as expected. *C. edule* indicated a prevalence of infection of 6.7% (n = 2/30) and a mean viral load of 1.1 x 10^1 copies/ µl of genomic DNA (range: <10 – 3.0 x 10^1 viral copies/ µl of genomic DNA).

During the laboratory trial, cumulative mortality of up to 100% was observed in *C. edule* in all three replicate experimental tanks within the first 4 days of the trial (n = 63). *C. gigas* showed low mortality up to Day 7 in all three experimental tanks (< 10%), after Day 7 mortality increased up to 66.7-100% (with a mean of 85.7% for all three experimental tanks) at the end of the trial (Figure 5). All *C. gigas* in the control tank were still alive at the end of the trial.

Oysters from the control tank (n = 30) were screened for OsHV-1 µVar by PCR on the last day (Day 14) of the trial and all individuals were negative for OsHV-1 µVar. In one experimental tank, on Day 3 and Day 4 OsHV-1 µVar was detected in both tissues in one dead cockle (Figure 5). *C. edule* prevalence of infection observed was 3.2% (n = 2/63) with an average viral DNA of 1.2 x 10^1 copies/µl (range: <10 - 1.2 x 10^4 viral copies/ µl of genomic DNA) of genomic DNA. In that same experimental tank, OsHV-1 µVar was detected in a single dead oyster on each of Days 5, 8, 11 and 13 (Figure 5). *C. gigas* had an overall OsHV-1 µVar prevalence of 4.4% (n = 4/90) with an average viral load of 3.0 x10^3 DNA viral copies/ µl (range: <10 - 1.2 x 10^4 viral copies/ µl of genomic DNA) of genomic DNA. In the other two replicate tanks, no positive signal of OsHV-1 µVar was detected in *C. gigas* or in the *C. edule*.

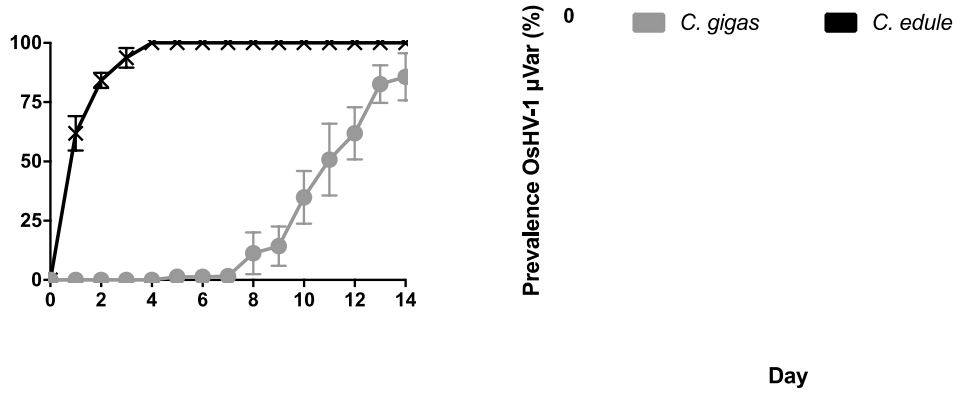


Figure 5. Cumulative mortality rates (A) and prevalence of OsHV-1 µVar (B) of C. gigas and C. edule of laboratory transmission trial

3.4. Discussion

In this study, the potential for an intertidal ecosystem engineer *C. edule* to become infected with and to act as an alternate host of OsHV-1 µvar was determined, through fied screening, laboratory transmission trials and using a variety of diagnostic methods (OIE 2017). The field survey demonstrated the ability of a virus to jump host to an alternate infaunal bivalve with potential further transmission through an ecosystem via trophic food webs. This suggests that OsHV-1 µVar is exhibiting plasticity, by spreading to other bivalves. In addition, the laboratory trial suggest that cockles could possibly act as a source of infection and transmit the virus back to oysters five days post exposure and at a low temperature of 14 °C, below the temperature (16 °C) considered necasarry for activation of the virus.

Herpesvirus infection was detected in the oysters being cultured at the sites in this study, but at a lower level compared to previous years in Ireland (Clegg et al. 2014; Morrissey et al. 2015; Prado-Alvarez et al. 2016). In this study oysters selectively bred for resistance to OsHV-1 µVar were used (oyster farmers Pers. Comm), which might be the main explanation for the lower prevalence in oysters. Moreover, temperatures were lower compared with previous years and rarely passed the temperature level (16 °C) considered necessary for the virus to become active, but the subsequent laboratory trial demonstrated the trial infection could occur at 14 °C. Survival of aquatic virus such as Abalone herpes virus outside their natural hosts, have been mostly observed at lower temperatures (Corbeil et al.

2012). In addition, a previous study investigating prevalence of OsHV-1 in *Ostrea edulis* observed a lower optimal temperature of 6 - 12 °C for *O. edulis*, compared to the threshold temperature of 16 °C in *C. gigas* (Feist 2008). In addition, a 14-fold OsHV-1 µVar replication was observed in mussels *Mytilus spp* at 13 °C under experimental conditions (O'Reilly et al. 2017). The findings of this study would suggest that OsHV-1 µVar may be generalist, adapting to new circumstances such as fewer available susceptible oysters due to the use of selectively bred oyster stocks more resistant to the virus, resulting in a "species jump" to another cohabiting bivalve species.

Infection was only investigated within surfaced cockles as it was considered more likely that surfaced cockles might be less healthy, potentially harbouring pathogens such as the virus and so more accessible to predators (Thomas & Poulin 1998) compared to their buried counterparts. However, surfaced cockles are associated with poor health (Blanchet et al. 2003) and are in direct contact with the water column and possible virus infected particles, therefore it might be that OsHV-1 µVar prevalence within *C. edule* communities might be an overestimation when buried individuals are taken into account. More individuals had higher prevalence of the virus in internal tissues compared with gill tissues indicating infection of cells and not just virus particles attached to external tissues such as gills. A possible explanation for higher internal infection rates could be that the virus particles are so small they may stick to feeding particles resulting in the virus being directed to the mouth and less likely to build up in the gills. A virosis was also observed in epithelial cells of the digestive gland tubules in cockles in Spain (Carrasco et al. 2011).

A high percentage of our cockles showed lower viral copies than the viral copy number of 10^4 mg^{-1} associated with oyster mortalities (Oden et al. 2011; Paul-Pont et al. 2013). This phenomenon was also found in a recent Australian study where low viral copies of OsHV-1 µVar were detected in other marine invertebrates; mussels, whelks and barnacles and it was suggested that these species could function as reservoirs and/or support transmission of OsHV-1 µVar in the environment (Evans et al. 2017). However, that study did not investigate if those infected invertebrates could transmit the virus to oysters. Of significance in our

study, however, was that a few individuals presented viral copy number of 10^4 mg^{-1} cockle tissue, which are considered to be high and associated with oyster mortalities, though the physiological impact of OsHV-1 µVar on *C. edule* was not investigated in this study.

A range of diagnostic methods plus a published in-house PCR, demonstrated that cockles carrier this virus. The DNA of the virus was quantified by qPCR, however it was not proven if the virus was able to replicate outside its host species in cockles over time. Herpesviruses can maintain themselves in low copy numbers and stay latent, and these infections with low viral DNA copies may not always develop to the next phase, an acute phase of infection (Sawtell 1997). Indeed, viral DNA and proteins have been found in oysters without showing full expression of the disease (Arzul et al. 2002; Petton et al. 2015). To understand if the virus is latent or viable outside its natural host it is essential to study virus replication over time. In oysters, virus replication has been successfully studied looking at viral RNA expression over time (Segarra et al. 2014a; Segarra et al. 2014b). However, the distribution of the virus in high copy levels in some instance in the cockles and the ability to transmit back to the oyster would indicate that the virus is viable and is replicating.

In the laboratory trial conducted in this study, OsHV-1 µVar was detected in naïve *C. gigas* 5 days post exposure. As the initial sample of *C. gigas* was negative for OsHV-1 µVar, these results indicate transmission of the virus from cockles to naïve oysters. A small number of *C. gigas* and all *C. edule* had died by the end of the trial, most likely due to the laboratory holding conditions with no sediment being present in the tanks (Richardson et al. 1993), and suboptimal conditions i.e. water quality as animals died. However, results from the transmission trial highlight that even when cockles have a low prevalence of the virus and when temperatures are below the threshold temperature for optimal replication and transmission of OsHV-1 µVar there is still the possibility of successful transmission of the virus from *C. edule* to *C. gigas* in the laboratory.

In this study, detection of OsHV-1 µVar did not show a significant correlation with cockle size, weight and age (number of shell rings), which indicated that all cockle cohorts were likely to have the virus present in their tissues. The consequence of

this possible ‘species jump’ could have larger ecological consequences. When cockles are consumed by mobile top predators, like bird species, those species could potentially functioning as new carriers, reservoirs or possibly new hosts of the virus. When birds migrate, the virus could extend its geographical range and be introduced to uninfected marine habitats. Certainly, previous studies indicated that birds contribute to geographic jumps of emerging infectious diseases and have been proven carriers of Lyme disease and Influenza A (Reed et al. 2003) and white spot disease in penaeid shrimps *Penaeus monodon* (Altizer et al. 2011).

The role of cockles as ecosystem engineers in viral disease transmission is poorly understood and therefore it is hard to forecast what effects this might have on possible transmission, infection and spatial extension of diseases in the marine ecosystem with subsequent wider trophic impacts. However, as an ecosystem engineer, particularly as an important food source for a number of species of birds, any negative impacts through increased mortality or reduced physiological ability in the cockle, are likely to have wider ecosystem impacts.

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Chapter 4.

The spread of the oyster pathogen *Vibrio aestuarianus* from a "hotspot" of infection in the marine environment

Abstract

Diseases and pathogens are very common in the marine environment and often result from complex pathogen-host-environment interactions. Bacteria *Vibrio aestuarianus* have recently been observed, associated with mortalities in the Pacific oyster *Crassostrea gigas* in a number of European countries. A better understanding of how *V. aestuarianus* is maintaining itself in the marine environment is needed to forecast possible disease outbreaks and to better manage the risks associated with its presence. Transmission between oysters at high density is likely, but the extent to which the bacteria can potentially spread to other species or at culture sites is not clear. Pacific oyster culture sites are often in intertidal areas with rich biodiversity and so may coexist with a lot of other marine invertebrates, who could potentially act as carriers or reservoirs of oyster pathogens or be themselves susceptible to that pathogen. In this study we investigated the presence of *V. aestuarianus* at two culture sites and its ability to spread outside this hotspot of infection. Two field studies were performed in 2015 and 2017. To assess if *V. aestuarianus* could be found outside of oysters, crabs and cockles found at the culture sites and having different ecological roles, were sampled and screened for the presence of *V. aestuarianus.* In addition to determine if *V. aestuarianus* could spread from culture sites (2 areas) and to what extent, sampling of wild mussels, which are widely distributed along the coast of Ireland, took place at different distances from the culture sites with an extension range of up to 30 km from these infection hotspots, to investigate the bacteriums' ability to spread in the marine environment. At the oyster culture sites, *V. aestuarianus* was observed in shore crabs *Carcinus maenas*, and edible cockles *Cerastoderma edule*. The bacterium was found in mussels *Mytulis spp*. up to 30 km from the endemic sites. Hydrodynamic models indicate that currents could distribute the pathogen far from the infection site and if potential reservoirs or carriers are available this can help to facilitate widespread distribution of the pathogen. This study provides a better understanding of how disease and pathogens can be spread within the

marine environment. The study would indicate that the use of hydrodynamic models will be needed to understand how oyster pathogens are being transported and transmitted in the marine environment and could inform the forecasting of future outbreaks and spread of disease.

Key words

Pacific oysters, *Crassostrea gigas, Carcinus maenas, Cerastoderma edule, Mytulis spp., Vibrio aestuarianus,* hydrodynamics

4.1. Introduction

Within host:pathogen interactions in marine systems, a range of other species, acting as carriers and reservoirs for pathogens, along with different trophic interactions may play a role in disease transmission (Lynch et al. 2007; Lynch et al. 2010; Small & Pagenkopp 2011; Carrasco et al. 2012). Pacific oysters, *Crassostrea gigas,* have faced a significant number of summer mortality incidents worldwide since the 1950*s'* (Lynch et al. 2012). These mortality events have been associated with a complex aetiology, including interactions between the physical status of the host, environmental parameters (such as temperature, exposure time and salinity) and the presence of diseases and pathogens such as ostreid herpes virus (OsHV-1) variants and more recently *Vibrio* spp. bacteria, including *Vibrio aestuarianus* (Samain & McCombie 2008; Sauvage et al. 2009; European Food Safety Authority 2010; Lynch et al. 2012).

Tison & Seidler (1983) first described *Vibrio aestuarianus subsp. aestuarianus (*ATCC®35048TM) after it was initially isolated from oysters by inoculating alkaline peptone broth and subsequently plating and isolating the organisms on thiosulfate-citrate-bile saltssucrose agar. In addition, related strains (83-98% relatedness) were observed within seawater, crabs and clams (Tison & Seidler 1983). This study was, however very limited, as no specific host species names, number of sampled individuals, prevalence of infection or information on its pathogenicity were provided. While *Vibrio aestuarianus subsp. aestuarianus (*ATCC®35048TM) was described as the reference strain by Tison & Seidler 1983 the French strain *V. aestuarianus subsp. francensis (*02/041) is used for French references (Garnier et al. 2008). Nowadays a large number of *V. aestuarianus*

strains have been detected (Garnier et al. 2007; Garnier et al. 2008; Saulnier et al. 2010; Goudenège et al. 2015). In Ireland *V. aestuarianus* strains 02/08; 03/08; S38/12/B; S15/13/C; S15/13/B; S14/14; S15/14 were detected during oyster mortalities from 2009-2014 (McCleary & Henshilwood 2015). A recent study indicated that regardless of differences in genetics, all strains showed the same disease processes (Travers et al. 2017). The bacteria can infect all life stages of oysters and mortality was observed across all ages (Garnier et al. 2007; Saulnier et al. 2010). However, adults appear to be more susceptible to *V. aestuarianus* compared to seed and juveniles (Dégremont et al. 2014; Travers et al. 2015; Green et al. 2016). The pathogenic impact of this bacteria was confirmed as laboratory trials showed mortalities in *C. gigas* after exposure to isolates of *V. aestuarianus* (Garnier et al. 2007; Saulnier et al. 2010; Azéma et al. 2017; Travers et al. 2017). Following a mass mortality in French oysters associated with *V. aestuarianus* in 2008 and the importation of French stocks of *C. gigas*, increased surveillance of *V. aestuarianus* prevalence in Irish oysters has been performed since 2011 (www[1]).

The role of *V. aestuarianus* has not fully been elucidated as it is also found at sites without mortalities in Spain (Romero et al. 2014) and Italy (Vezzulli et al. 2014). Much remains unknown about the processes of infection and the disease dynamics within oyster populations. In 2007, 19 different strains of *V. aestuarianus* were isolated from French oysters (Saulnier et al. 2010). In subsequent years *V. aestuarianus* has spread throughout Europe and it has been isolated from oysters during mortality events not only in France (Garnier et al. 2007; Saulnier et al. 2010) but also Ireland (McCleary & Hensilwood 2015), Italy (Domeneghetti et al. 2014), Scotland (EFSA 2015) and New Zealand (Keeling et al. 2014). A number of *Vibrio* spp including *V. aestuarianus* but also *V. splendidus, V. splendidus-like,* and *V. gallicus* have all been detected in dying Pacific Oysters in Ireland (European Food Safety Authority 2010). Though many species of Vibrios are associated with diseased shellfish, the main pathogenic species associated with mortalities are *V. splendidus* (Garnier et al. 2007; Saulnier et al. 2009; Saulnier et al. 2010) and *V. aestuarianus* (Le Roux et al. 2002; Gay et al. 2004; Saulnier et al. 2010). In Ireland, mortalities associated with *V. aestuarianus* have been mainly observed in early Autumn in September and October and variable prevalence of

infection were observed between sites (Annual NRL meeting on 28-30th of March 2017).

A range of species may act as carriers and reservoirs for pathogens and trophic interactions may also play a role in disease transmission (Lynch et al. 2007; Lynch et al. 2010; Small & Pagenkopp 2011). Regarding *V. aestuarianus* there is not much known how this bacterium is sustaining itself outside the host, but *V. aestuarianus* was detected in the environment; in the sediment up to 30 meters from an oyster culture site (Azandégbé et al. 2010) and in sediment and plankton samples in laboratory experiments (Vezzulli et al. 2014). The presence of *Vibrio spp.* and *V. aestuarianus* in sediment around Pacific oyster culture sites was positively correlated with temperature (Thompson et al. 2004; Azandégbé et al. 2010; Romero et al. 2014). *V. aestuarianus*, was detected in low intensities during the winter and Azandégbé et al. 2010 suggested it could be a permanent presence in the environment. Indeed, a more recent study investigating the ecology of *V. aestuarianus* showed that *V. aestuarianus* 01/32 strain maintained viability in the sediment over a long time span suggesting persistence in the environment outside the host (Vezzulli et al. 2014). Recently *V. aestuarianus* strains were isolated from the Mediterranean mussels *Mytilus galloprovincialis* at sites close to aquaculture facilities and natural Pacific oyster reefs in Italy. No mortalities were observed in infected mussels from the field, while moderate pathogenicity in mussels were observed under experimental conditions (Romero et al. 2014).

In Ireland, invertebrate species such as the green shore crab *Carcinus maenas,* the common cockle *Cerastoderma edule* and mussels *Mytilus spp*. are living in intertidal areas (Longshaw & Malham 2013; Amaral & Paula 2007; Lynch et al. 2014) and are often observed at Pacific oyster culture sites in Ireland. Those species have been identified as key species within the marine communities, each with their own feeding mechanisms, distribution patterns and ecosystem roles within the marine environment. *C. maenas* is a mobile scavenger, that feeds on oysters (Lovely et al. 2015), as well as other intertidal organisms and it has been suggested that these crabs can migrate up to 600 meters in intertidal areas within a single tide (Holsman et al. 2006). Of significance, *C. maenas* acts as an intermediate host to a number of parasites and may function as a source of

infection (Torchin et al. 2001). *C. maenas* may increase the geographical range of diseases and pathogens directly by the introduction of diseases and pathogens to new ecosystems when it migrates, and indirectly by transmitting pathogens to predators including birds, fish species and mammals (Bush et al. 1993; Lotz et al. 1995). *C. edule* is an ecosystem engineer (Longshaw & Malham 2013) and an important species within many marine food webs (Eriksson et al. 2010; Lotze et al. 2006; Cesar 2009). In addition, *C. edule,* especially surfaced individuals, are host to a range of pathogens and diseases (Blanchet et al. 2003) and are often a first or a second intermediate host for a range of trematodes (de Montaudouin et al. 2009; de Montaudouin et al. 2012; Tuntiwaranuruk et al. 2004; Morgan et al. 2012). As a common food source for mobile predators like fish and birds (Goss-Custard & Jones 1976; Phil 1985; Beukema 1992;Ponsero et al. 2009), cockles also play a significant role in pathogen transmission and pathogen distribution via their predators (Mouritsen & Poulin 2010). *M. galloprovincialis* has been associated with *Vibrio aestuarianus* in Italy (Romero et al. 2014). In Ireland, *Mytilus* spp. including the blue mussel *M. edulis*, *M. galloprovincialis* and the hybrids of both parent species are present. The east coast of Ireland is exclusive to *M. edulis* while *M. edulis*, *M. galloprovincialis* and hybrids are widely found along the southern, western and northern Irish coastline (Lynch et al. 2014). In Ireland, the mussel industry is a significant segment of the aquaculture sector (FAO 2017) and some mussel culture sites will have co-culture with Pacific oysters (Marine Institute report 2013), which could support interspecific interactions. *Mytilus spp*. similar to *C. edule* are described as ecosystem engineer by their ecological importance in the marine ecosystem; they can form natural reefs that can enhance species biodiversity (Guenther 1996; Beadman et al. 2004), supply a link between benthic and pelagic levels through their efficient filter feeding (Prins & Smaal 1994; Beadman et al. 2004), are a food source for starfish, crabs like *C. maenas*, gastropods (Spencer 2002) and a wide variety of seabirds (Ens & Alting. 1996; Caldow et al. 2003) and have been associated with a wide variety of parasites (Crowley 1972; Lynch et al. 2014). Since *C. maenas*, *C. edule* and *Mytilus spp.* have all been associated with a variety of pathogens and diseases and due to their varying ecological roles, there is the potential for these species to support the spread of *V. aestuarianus*, potentially in different ways if they could act as carriers or reservoirs. These species have been defined as carriers and reservoirs of oyster

pathogen Ostreid herpesvirus-1 microvar (O'Reilly et al. 2017, Bookelaar et al. Chapter 2), which makes them an interesting species to investigate the maintenance and spread of *V. aestuarianus* in the marine environment.

Water currents can transport all sized particles over long distances and therefore also extend their natural geographical range (Murray et al. 2008). For the south east coast of Ireland; water comes in from the Celtic Sea moving northwards up to St Georges Channel to turn southwards and following the coast line of Ireland clockwise (Figure 1) (Hill et al. 2008). Dungarvan Bay is described as an oceanic environment where the water residence time (water flow of 3.68 m^3s^{-1}) is long (>10 days) and the water column is mixed very well. The susceptibility of this system is high, due to long retention and low dilution potential. It is an open bay with local differences in nutrients resulting in patchy growth rates within oysters at the culture site (Pers Comm. with Ben Dallaghan BIM; UISCE project report material BIM. 2007). The currents at the coast line around Dungarvan are limited (Pers Comm. with Nicolas Chopin from the MI).



Figure 1. Main ocean current pathways (Adapted from Hill et al. 2008)

Flushing rate is low in Dungarvan Bay and therefore it is possible that pathogens such as *V. aestuarianus* might be retained for several days in the bay if not for longer. Animals in the bay are high likely to filter particles several times thus increasing their risk of infection. The water currents could facilitate direction and spread of free infected particles. A well-known example is the bacteria *Vibrio cholera*, a common disease in the Middle East and Africa since the 1970's, which reached South America in the 1990's by two routes (1) ballast water from ships coming from infected areas and (2) El Niño currents which may have transported *V. cholera* infected sediment particles and zooplankton (Tamplin & Parodi 1991; Glass et al. 1992). With a focus on oyster pathogens, OsHV-1 μvar has been detected up to 26 km from an OsHV-1 μVar endemic *C. gigas* culture site outside its natural host in *Mytulis spp.* (O'Reilly et al. 2017). Disease and pathogen specific hydrodynamic and biological models are required to forecast possible distribution patterns of pathogens in the marine environment (Murray et al. 2008).

In this study, the initial aim was to investigate if the Pacific oyster pathogen *V. aestuarianus* could be found outside its host *Crassostrea gigas* in other marine invertebrates at Pacific oyster culture sites in Ireland. Previous research detected *V. aestuarianus* in mussel species *M. galloprovincialis* but only close to aquaculture practices or Pacific oyster sites. Therefore, our second objective was to investigate if *V. aestuarianus* could be found in mussels (a mixture of two species and hybrids) in Ireland, and then using the mussel due to its wide distribution to determine how far outside the hotspot of infection at culture sites, the bacterium can spread in the marine environment, taking hydrodynamic models of water movements where available into account.

4.2. Material and methods

In this study two field studies were carried out.

(1) Field study 1. Vibrio aestuarianus screening of invertebrate species the shore crab Carcinus maenas and edible cockle Cerastoderma edule

The first field study investigated the potential of *V. aestuarianus* to infect other invertebrates by screening mobile scavenger *Carcinus maenas* and suspension feeder *Cerastoderma edule* collected at two Irish Pacific oyster culture sites

Dungarvan and Carlingford Lough in spring and summer (April till August) of 2015. *V. aestuarianus* has been detected in oysters at both sites (Pers. Comm. O'Toole at Annual NRL meeting on 28-30th of March 2017; NRL report 2017).

Study sites

Sampling of crabs and cockles took place at high shore (approx. 500 m from oyster trestles) and at trestles at Pacific oyster culture sites in Dungarvan (52.0936 °N - 7.6204°W) and Carlingford Lough (54.0733°N -6.1994°W), approximately 245 km apart (Figure 2). The culture site in Dungarvan has been recognized as a *Vibrio aestuarianus* endemic site, while *V. aestuarianus* has been detected but much more sporadically in Carlingford Lough (Pers. Comm. with Marine Institute). Sites were sampled eight times over spring and summer, from April to August 2015.

The two culture sites between them are responsible for the majority of Pacific oyster production in Ireland, but both ecosystems are very different in their geographical, ecological and hydrodynamic characteristics. This results in different current movements, tidal exchange etc. which will have different effects on spread of pathogens and disease dynamics. The oyster culture site in Dungarvan is sheltered, being almost closed off by the linear Cunnigar spit to the east (www[2]). Intertidal habitats are dominated by sandflats and it has mudflats at the edge of saltmarsh habitats. The water quality of Dungarvan Harbour varies from moderate to good, representing unpolluted water and acceptable levels of biochemical oxygen demand (EPA 2015). Carlingford Lough has a gravelly substrate covered by 3-5cm of muddy silt is fed by the Newry River, and has generally shallow waters of 2-5 m. Water quality within the lough is good; mean salinity is 32.5 and the annual temperature varies between 3 - 20°C (www[3]).

Macroinvertebrate sampling

Green shore crabs *Carcinus maenas* (n=162 in total) were randomly collected at the trestles and at the high shore in Dungarvan (n=75) from April to August 2015. In Dungarvan, no *C. maenas* was found around the trestles and individuals were collected from inside oyster trestles bags. Numbers of *C. maenas* collected per month, location and site varied between 2 up to 18 (Table 1).

Table 1. C. maenas collected at Dungarvan and Carlingford Lough

Site	Location	Sample month	Number *C. maenas* collected
Dungarvan	High Shore	April	4
	Trestles	April	3
Carlingford Lough	High Shore	April	3
	Trestle	April	5
Dungarvan	High Shore	May	2
	Trestles	May	7
Carlingford Lough	High Shore	May	11
	Trestle	May	7
Dungarvan	High Shore	June	7
	Trestles	June	12
Carlingford Lough	High Shore	June	11
	Trestles	June	13
Dungarvan	High Shore	July	10
	Trestles	July	18
Carlingford Lough	High Shore	July	12
	Trestles	July	12
Dungarvan	High Shore	August	4
	Trestles	August	8
Carlingford Lough	High Shore	August	7
	Trestles	August	6

Surfacing edible cockles *Cerastoderma edule* (105) were randomly collected at the high shore in Dungarvan (n= 38) and at the high shore and oyster trestles at Carlingford Lough (n= 66). In Dungarvan, no *C. edule* were found around the trestles and within oyster trestles bags. Numbers of *C. edule* collected per month, location and site varied between 2 up to 13 (Table 2).

Table 2. C. edule collected at Dungarvan and Carlingford Lough

Site	Location	Sample month	Number *C. edule* collected
Dungarvan	High Shore	April	3
	Trestles	April	-
Carlingford Lough	High Shore	April	2
	Trestles	April	4
Dungarvan	High Shore	May	12
	Trestles	May	-
Carlingford Lough	High Shore	May	7
	Trestles	May	8
Dungarvan	High Shore	June	10
	Trestles	June	-
Carlingford Lough	High Shore	June	8
	Trestles	June	9
Dungarvan	High Shore	July	9
	Trestles	July	-
Carlingford Lough	High Shore	July	10
	Trestles	July	13
Dungarvan	High Shore	August	4
	Trestles	August	-
Carlingford Lough	High Shore	August	3
	Trestles	August	6

Crabs and cockles were returned to the laboratory where their *w*eight (g) and length/carapace width (mm) of invertebrates were recorded using a balance scales and vernier calipers. Gill tissues were removed for DNA extraction.

(2) Field study 2. Vibrio aestuarianus screening of mussels Mytilus spp.

The second field study carried out in September 2017, investigated the potential range extension of *V. aestuarianus* from the hotspot of infection at the oyster culture site (Sample site 1). Mussels were collected at the oyster culture site in Dungarvan (Sample site 1), where *V. aestuarianus* is regularly detected in oysters (Pers. Comm. With Marine Institute), and at multiple sites radiating out from the culture site, with an extension range up to 30 kilometers (sample sites 2-8) (Figure 2, Table 3). Additionally, mussels (sample sites 9-10) close to another culture site in Cork harbour, which have been associated with the presence of *V. aestuarianus* and *C. gigas* mortalities (Marine Institute Pers. Comm.), were also screened for

the presence of *V. aestuarianus* in October 2017. These studies were up to 10 km from the culture site (not sampled at sample sites itself) (Figure 2, Table 4).

In Dugarvan Bay, taking into account the main ocean current pathways, it is expected that water movement would occur from Sample sites 6, 5 and 4 before reaching the culture site (Sample site 1). After reaching the main culture site, water currents would move in the direction of Sample sites 2, 3, 7 and 8 ((Pers Comm. with Ben Dallaghan BIM; Pers Comm. with Nicolas Chopin from the Marine Institue).

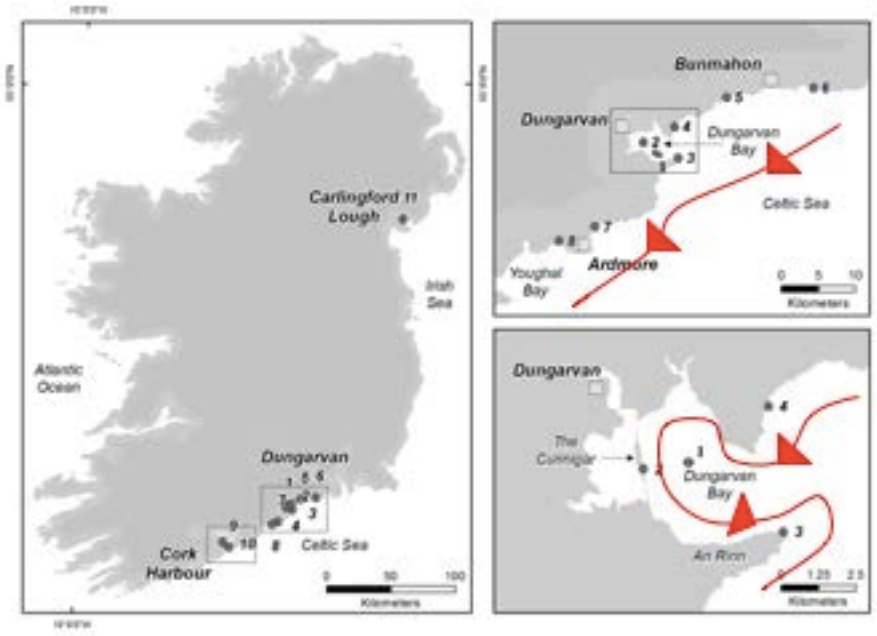


Figure 2. Crassostrea gigas culture site at Dungarvan, Co. Waterford and Carlingford Lough, Co. Louth, Ireland.

Table 3. Details of the eight sample sites for mussels around Dungarvan Bay

Sample site	Characteristics	Coordinates
1 Trestles	main oyster culture site in Dungarvan	52.0936°N - 7.6204°W
2 High Shore	approximately 500 m from sample site 1	52.0736°N - 7.6067°W
3 Helvic Head	approximately 4 km south from sample site 1	52.0544°N - 7.5384°W
4 Clonea Beach	approximately 4 km north from sample site 1	52.0923°N - 7.5453°W
5 Ballyvooney Cove	approximately 10 km north from sample site 1	52.1273°N - 7.4424°W
6 Annestown Beach	approximately 20 km north from sample site 1	52.1380°N - 7.2716°W
7 Ballyquin Beach	approximately 20 km south from sample site 1	51.9713°N - 7.7025°W
8 Whiting Bay	approximately 30 km south from sample site 1	51.9543°N - 7.7728°W

Table 4. Details of the two sample sites for mussels around Cork habrour

Sample site	Characteristics	Coorodinates
9 Rochespoint	approximately 10 km from culture site in Cork harbour	51.7934°N - 8.2543°W
10 Ringaskiddy	approximately 10 km from culture site in Cork harbour	51.8307°N - 8.3239°W

(3) Processing of oysters and mussels

To investigate the infection at the hotspot in Dungarvan, half grown (n = 30) and adults (n = 30) of host species *Crassostrea gigas* were collected at the trestles (Sample site 1) in June 2017 and September 2017 (n = 120 oysters in total) to screen for *V. aestuarianus* levels.

Sampling of the mussels took place from July to October. In July 2017 an initial sampling of blue mussel *Mytilus spp.* took place and mussels were collected randomly on the shore at sample site 3 (n = 60) and at sample site 4 (n = 22) to confirm presence of the bacterium. In September 2017 sampling of *Mytilus spp*. took place at Sample sites 1-8 sites (n = 30 per sample site). Sampling of *Mytilus spp*. took place at Rochespoint (n = 30), (sample site 9) and Ringaskiddy (n = 30)

(sample site 10) in early Oct 2017. Mussels at the main oyster culture site were sampled directly from the oyster bags on the trestles approximately 1 foot above the sediment. At other sample sites mussels were collected randomly at the shores. In total, 382 mussels were screened for *V. aestuarianus*.

Mussels and oysters were returned to the laboratory where their whole wet weight (g) and length/carapace width/length (mm) of invertebrates were recorded using a balance scales and vernier calipers. A piece of gill tissues (approx. $5mm^2$) was removed for DNA extraction.

(4) Molecular diagnostic screening

DNA extraction

Gill tissues of the invertebrates (crabs, cockles, mussels and oysters) were removed and washed in double deionized water (ddH_2O) thoroughly and blot dried using tissue paper. DNA extraction of tissue samples (approx. $5mm^2$) was performed using the Chelex-100 methodology (Walsh et al. 1991). Tissue samples from the invertebrates were placed in a 10% chelex solution (100 microlitres volume) (Sigma Aldrich) and placed in a thermo Hybaid thermal cycler for 1 hour and 10 minutes heated at 99°C to facilitate cell lysis (Walsh et al. 1991).

Polymerase chain reaction (qPCR) for Vibrio aestuarianus

qPCR is performed for detection of multiple strains of *V. aestuarianus* (Irish and French strains) following the protocol of McCleary and Henshilwood 2015 (McCleary & Henshilwood 2015). A total of 5 µl of DNA per invertebrate was used to detect *V. aestuarianus* prevalence. The master mix for qPCR consisted of a total volume of 20 µl; 5.62 µl dH_2O ,0.75 µl of 5 *µM* forward primer (dnaJ f420), 0.75 µl of 5 *µM* reverse primer *(dnaJ r456))*, 0.38 µl of 5 *µM* of probe (*dnaJ* p441) and 12.5 µl of 2x Taqman mastermix. All samples were performed in duplicate. Negative controls (n = 2) of 5 µl double distilled water (ddH_2O) were used. Positives controls (n = 2) contained 5 µl *V. aestuarianus* infected DNA. A tested sample is considered positive if its mean Ct value is below 37.

Polymerase chain reaction (qPCR) for Mytilus species identification

The Me15 / Me16 primer pair (Me15 (5'-CCAGTATACAAACCTGTGAAGA-3'), 0.5µl of the primer Me16 (5'-TGTTGTCTTAATGGTTTGTAAGA-3') was used to distinguish between *M. edulis* and *M.galloprovincialis* species as well as their hybrid spp (Inoue et al. 1995). A modified PCR mastermix was used. The mastermix contained 14µl of ddH2O, 5µl 5x green buffer solution, 2.5µl dNTP, 1.5µl MgCl2, 0.5µl each of the forward primer (Me15) and the reverse primer (Me16),1µl Taq polymerases and 2µl of DNA. The conditions for PCR were 94º C for 30s, 55º C for 45s and 70º C for 90s, this was completed for 40 cycles (Inoue et al., 1995).

The gel was made up of 5ml 20x TPE buffer, 220ml distilled water, 4g of 2% agarose powder and 22µl of Syber Safe DNA used to stain the gel. The buffer solution consisted of 50ml of 20x TPE buffer into a 2000 ml graduated cylinder, filled with distilled water. The gel was left to run for approximately 50 minutes to an hour. In order to see the bands that were amplified, UV light was needed. These gels were placed under UV to show the product size expected for the mussel species are 126bp for *M. galloprovincialis*, 180bp for *M. edulis* and both base pairs for hybrids (Suchanek et al. 1997)

(5) Statistical analyses

Shapiro Wilke test was used to test for normality Kruskal Wallis tests were used to test differences in weight, length and width between sites.. Chi square test were used to test for difference in prevalence of *V. aestuarianus* between sites. Pearsons Chi-squared tests were used to test for differences of prevalence of *V. aestuarianus* for average weight, width and length of mussels per site. For all analysis, a critical value of 0.05 was used to confirm significant results. Weight and lengths are presented as average ± standard deviation and with a range of minimum and maximum values.

4.3. Results

(1) Field study 1. Vibrio aestuarianus screening of invertebrate shore crab Carcinus maenas and edible cockle Cerastoderma edule

Shore crab Carcinus maenas

Crabs had an average weight of 8.1 ± 9.7 gram (range: 0.3 - 45.9 gram) and an average carapace width of 29.4 ± 11.2 mm (range: 10.6 - 61.7 mm). *V. aestuarianus* was detected in *Carcinus maenas*, at both Dungarvan (Table 3) and Carlingford Lough (Table 4) *V. aestuarianus* was detected in 22 individuals out of the 162 (13.6%) screened. CT values for *V. aestuarianus* (average of duplicate per sample) ranged from 24.66 to 36.83. At Dungarvan, *V. aestuarianus* detection was observed from May to August and varied from 0-75%. *V. aestuarianus* was only detected in the *C. maenas* collected at the trestles (Figure 3) not at the high shore. At Carlingford, *V. aestuarianus* detection was only observed in one individual collected at the high shore in July (Figure 4).

Table 5. Prevalence of Vibrio aestuarianus per shore location detected from gill tissue of C. maenas in Dungarvan

Location	**Number *C. maenas* screened**	**% Positive**	**CT Value average/ range**
High Shore	27	0%	-
Trestle	48	43.8% (n=21)	33.5 / 24.7-36.8

Table 6. Prevalence of Vibrio aestuarianus per location and month for gill tissue of C. maenas in Carlingford Lough

Location	**Number *C. maenas* screened**	**% Positive**	**CT Value average/ range**
High Shore	44	2.3%	36.8
Trestle	43	0%	-

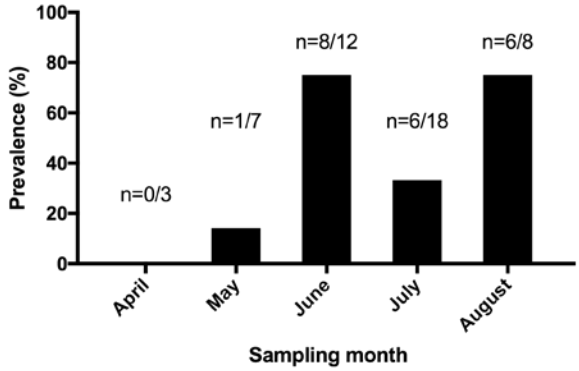


Figure 3. Prevalence Vibrio aestuarianus in C. maenas at trestles in Dungarvan

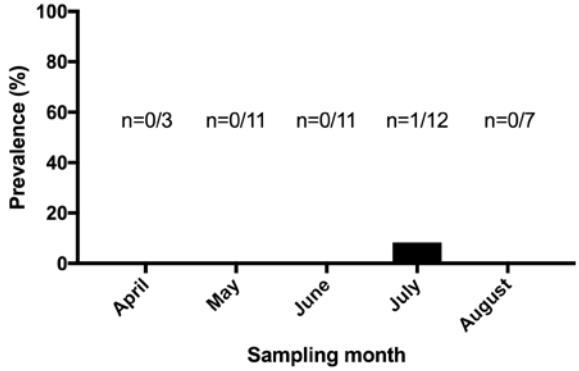


Figure 4. Prevalence Vibrio aestuarianus in C. maenas at high shore in Carlingford Lough

Cockles had an average weight of 10.0 ± 6.2 gram (range: 0.3 – 21.9 gram) and a width of 26.1 ± 7.0 mm (range: 9.3 – 36.3 mm). Only two individual *C. edule* out of 105 sampled (1.9%) had a positive detection for *V. aestuarianus.* Both positive samples from *C. edule* were collected at the high shore in Dungarvan (Table 7) at the end of July and mid-August (Figure 5) and CT values of *V. aestuarianus* ranged from 35.2 and 36.5. No table is included for Carlingford Lough due to all negative results.

Table 7. Prevalence of Vibrio aestuarianus, location and month for gill tissue of C. edule in Dungarvan

Location	Number *C. edule screened*	% Positive	CT Value average/ range
High Shore	38	5.3% (n=2)	35.9/ 35.2-36.5
Trestle	-	-	-

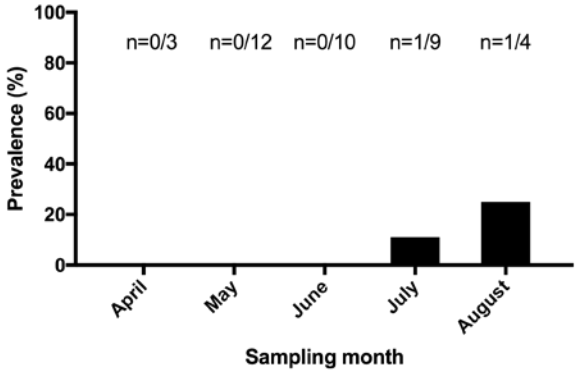


Figure 5. Prevalence Vibrio aestuarianus in C. edule at high shore in Dungarvan

(2) Field Study 2. Vibrio aestuarianus screening of the blue mussel Mytilus spp. at radiating distances from the culture site

Screening of Crassostrea gigas

On the 23rd of June 2017, half-grown oysters screened from Dungarvan showed a prevalence of 86.7% (n = 26/30) of *V. aestuarianus* with an average CT of 31.2 (range: 25.4 - 36.9) Adults showed a lower prevalence of *V. aestuarianus* at 73.3% (n = 22/30) and an average CT of 32.8 (range: 25.1 - 36.6). On the 6th of September, prevalence for both age classes was lower compared with previous sampling time. Half-grown oysters had a prevalence *V. aestuarianus* of 6.7% (n = 2/30) with an average CT of 33.3 (range: 19.9 - 36.2). This time adults had a slightly higher prevalence *V. aestuarianus* at 20% (n = 6/20) with an average CT of 31.5 (range: 23.6 - 36.9) (Figure 6).

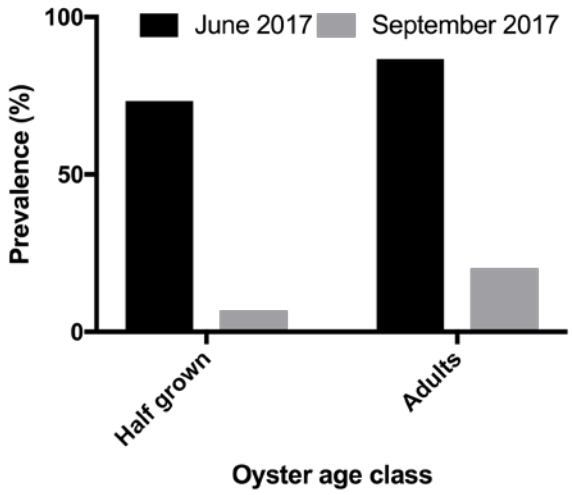


Figure 6. Prevalence of V. aestuarianus in half grown and adult oysters in June and September 2017 (n=30 for each oyster age class at each sample date)

Screening of Mytilus spp.

On July 18th 2017, an initial sample of mussels was taken at Clonea beach (n = 22) and Helvic Head (n = 60) and screened. At Clonea beach all mussels were free of *V. aestuarianus* (n = 0/22), while 13.33% (n = 8/60, CT value average: 36.0, CT value range: 33.1 - 36.9) of the mussels showed prevalence of *V. aestuarianus* at Helvic Head.

On the 6th and 7th September 2017 sampling took place at the Pacific oyster site (trestles) and high shore in Dungarvan and at Sample sites 3-8 (n=30 mussels per site). Individuals collected were heavier and larger at the trestles compared with other sites (Table 8).

Table 8. Weight (gram), length (mm) and width (mm) of mussels for each sample site (n=30)

Sample site	Average weight	Weight range	Average length	Length range	Average width	Width range
1	5.3±3.8	0.8-19.6	34.3±8.1	19.2-53.7	20.2±4.1	13.1-29.1
2	1.6±1.5	0.4-6.9	21.8±5.9	13.8-39.4	14.5±4.2	8.3-23.0
3	2.2±2.0	0.4-9.3	22.3±7.0	12.3-39.3	12.2±3.7	6.9-18.6
4	3.0±2.1	0.6-8.2	24.4±6.3	14.9-37.4	13.6±3.4	7.1-20.3
5	0.7±0.4	0.2-1.7	15.1±3.4	7.2-22.4	8.0±1.5	5.4-11.9
6	0.7±0.6	0.1-2.7	13.9±4.4	7.0-25.7	7.7±2.3	4.1-14.3
7	1.3±1.2	0.3-6.7	19.1±5.6	12.4-39.3	9.9±3.6	6.1-19.3
8	0.6±0.4	0.1-1.8	15.2±3.7	9.2-26.1	7.8±2.2	5.3-17.3

**Sample site 1: Trestles, 2: High Shore, 3: Helvic head, 4: Clonea Beach, 5: Ballyvooney Cove, 6: Annestown beach, 7: Ballyquin beach, 8: Whiting Bay*

Prevalence of *V. aestuarianus* was observed at all sites, varying from 6.7% up to 53.3%. A significantly higher prevalence of *V. aestuarianus*, 53.3%, was observed at the trestles at the main Pacific oyster culture site ($X^2 = 29.4$, df = 7, $P < 0.01$). No pattern between distance outside culture site in Dungarvan and prevalence of *V. aestuarianus* was observed. Lowest prevalence was observed at Helvic Head (Sample site 3) (6.7%), only 4 km away from the trestles in Dungarvan. After trestles in Dungarvan highest prevalence of *V. aestuarianus* was observed at Ballyquin beach (Sample site 7) (26.7%) which is 20 kilometers south from the trestles in Dungarvan (Table 9 & Figure 7). CT values were relatively consistent between sites.

Table 9. Prevalence of Vibrio aestuarianus of mussels for sample sites 1-8

Sample date	Sample site	Numbers	Prevalence of *V. aes*	CT Value average/ range
6-9-2017	1 Trestles	30	53.3%	35.7 / 33.7-36.6
6-9-2017	2 High Shore	30	10.0%	36.3 / 35.9-36.5
6-9-2017	3 Helvic Head	30	6.7%	35.2 / 34.0-36.5
7-9-2017	4 Clonea Beach	30	16.7%	36.1 / 35.4-36.8
7-9-2017	5 Ballyvooney Cove	30	16.7%	36.5 / 35.8-36.9
7-9-2017	6 Annestown Beach	30	13.3%	35.8 / 34.7-36.5
7-9-2017	7 Ballyquin Beach	30	26.7%	35.4 / 32.2-36.5
7-9-2017	8 Whiting Bay	30	16.7%	36.3 / 36.3-36.9

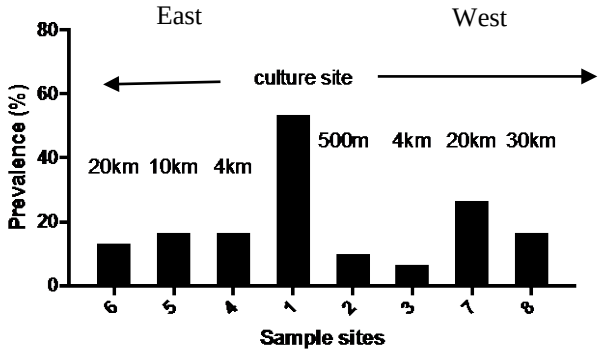


Figure 7. Prevalence Vibrio aestuarianus in mussels at sample sites 1-8

Again, prevalence of *V. aestuarianus* was also observed at the sample sites Ringaskiddy (13.3%) and Rochespoint (20%) on the 6th of October (Table 10). No data is available on morphometrics for these sample sites.

Table 10. Prevalence of Vibrio aestuarianus of mussels for sample sites 9 and 10 (October 2017)

Sample date	**Sample site**	**Numbers**	**Prevalence of *V. aes***	**CT Value average/ range**
6-10-2017	9 Ringaskiddy	30	13.3%	34.8/ 32.5-36.7
6-10-2017	10 Rochespoint	30	20.0%	35.8 / 33.3-36.8

Prevalence of *V. aestuarianus* was significantly higher ($X^2 = 4.1$, df= 1, $P < 0.05$) in September (16.7%) compared with July (0%) at Clonea Beach, while a slightly but not significantly higher ($X^2 = 0.9$, df= 1, $P > 0.05$) prevalence was observed in July (13.3%) compared with September (6.7%) at Helvic Head.

A significant correlation was observed between average weight and prevalence, with higher prevalence for sites with heavier individuals ($Y = 6.762*X + 6.996$, $R^2= 0.5419$, $P < 0.05$). No significant relationship was observed between average length and prevalence ($Y= 1.534*X - 11.84$, $R^2= 0.4881$, $P = 0.054$) and average width and prevalence ($Y= 2.135*X - 5.043$, $R^2= 0.3991$, $P = 0.092$) (Figure 8).

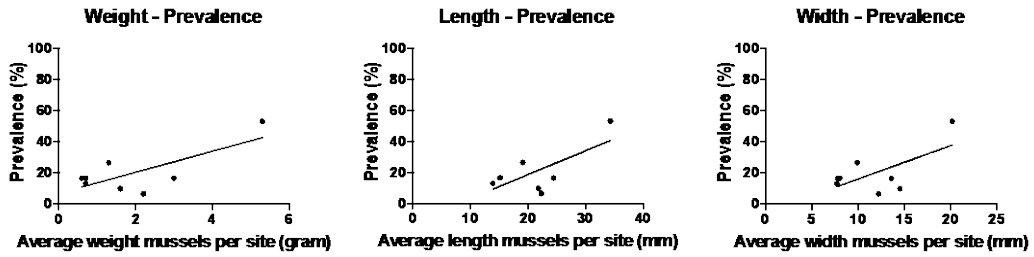


Figure 8. Correlation between average weight, length and width of mussels per site and prevalence of Vibrio aestuarianus

Coghlan et al. (2018) investigated the species id of *Mytulis spp.* of a subsample (n = 163) collected during this study. For Sample sites 1-8 overall, *Mytulis spp*. composition was 57% *M. edulis*, 20% *M. galloprovincialis* and 23% hybrids (see Figure 9 for species ID for each site) (Coghlan et al. 2018).

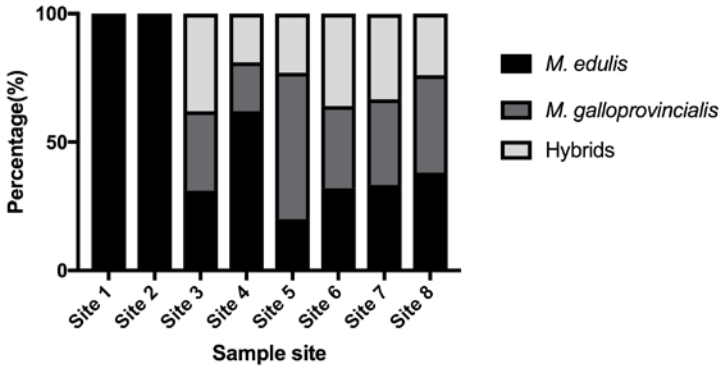


Figure 9. Mytulis spp. id per site

Prevalence of *V. aestuarianus* was highest in *M. edulis* with 23.6% (n = 21/89) followed by hybrids 21.9% (n = 7/32), and lowest in *M. galloprovincialis* with 11.9% (n = 5/42), however these differences were not significantly different (X^2 = 2.5, df= 2, P = 0.29).

4.4. Discussion

Vibrio aestuarianus is a pathogen associated with mortalities in Pacific oysters (Le Roux et al. 2002; Gay et al. 2004; Saulnier et al. 2010), which has possibly spread throughout Europe with movements of oysters for culture and via ship ballast water (Martinez-Urtaza et al. 2008).

To our knowledge no *V. aestuarianus* screening has been performed in invertebrates outside endemic sites (Pacific oyster culture sites) in Ireland. This study detected *V. aestuarianus* in wild *C. maenas* at both Pacific oyster cultures sites Dungarvan and Carlingford Lough, in wild *C. edule* at Pacific oyster culture site in Dungarvan and in wild *Mytilus spp.* (*M. edulis*, *M. galloprovincialis* and hybrids) at Pacific oyster culture site in Dungarvan and up to 30 km outside the endemic sites. To the best of our knowledge *V. aestuarianus* has previously only been detected in the *M. galloprovincialis* in Spain (Romero et al. 2014) apart from *C. gigas*, therefore this is the first study detecting *V. aestuarianus* in wild *C. maenas*, *C. edule*, *M. edulis* and *Mytilus* hybrids. This phenomen can help to understand how *V. aestuarianus* could spread in the environment.

A study investigating the seasonality and distribution of *V. aestuarianus* did not find a significant difference in concertation of *V. aestuarianus* in sediment samples collected under *C. gigas* trestles and 30 meters away from the trestles (Azandégbé et al. 2010). These results are suggesting that hydrodynamics play an important role in the distribution patterns of this bacterium. We did see a significant difference in prevalence of *V. aestuarianus* in *Mytulis* spp. between the main culture site in Dungarvan and other sample sites. However, no significant difference in prevalence in *V. aestuarianus* between the other sample sites were observed. These results imply that the bacterium can be maintained for a long time outside its known host (Azandégbé et al. 2010) and possible might be replicating on the mussel. In the furure screening of water samples (not performed in this study) could help to give better understanding of distribution patterns of this bacterium.

Currents around Dungarvan Bay are limited (Pers. Comm. with Nicolas Chopin from BIM) and therefore this pathogen was not expected to be dispersed to sites outside Dungarvan Bay. Also, although *V. aesturarianus* showed longer viability in sediments and its ability to attach to chitin particles and copepods (Vezzulli et al. 2014) it is highly affected by environmental factors like pH, salinity and oxygen (Eiler et al. 2006; Vezzulli et al. 2014). However, there is not much known about the viability of the bacterium in the water.

The pathogen was observed far outside our endemic study site; in mussels 30 km from the culture site in Dungarvan Bay and in mussels 10 km from in the Cork Harbour culture site, suggesting that this pathogen could be spread by water movements. Taking the main ocean current pathways of Ireland into consideration, it was not initially expected to detect *V. aestuarianus* in mussels at sites (Sites 4: Clonea Beach, 5: Ballyvooney Cove and 6: Annestown) eastward from Dungarvan. Moreover, the highest prevalence outside our endemic study site was observed almost 20 kilometers west of the endemic site (Site 7: Ballyquin Beach). Possible explanations of prevalence of *V. aestuarianus* in mussels at these sites could be disease spread from smaller endemic Pacific oyster cultures site in Woodstown (52.1474°N, -6.9869°W) 20 km eastwards from Annestown and Ballymacoda (51.8911°N, -7.9245 °W) 20 from Ballyquin Beach. These results display the complexity of spread of disease in the marine environment and more research will be needed to understand how this bacterium is transporting within the marine environment. Though, besides water currents, other mechanisms might be responsible for spread of *Vibrio aestuarianus* in mussels such as horizontal transmission (Azéma et al. 2015) from mussel to mussel.

Of significance, a higher prevalence *V. aestuarianus* was detected in *Mytilus spp*. (53.3%) compared to half grown (6.7%) and adult (20%) oysters collected at the trestles in Dungarvan. However, qPCR CT levels were higher for *Mytilus spp*. compared to both half grown and adult oysters, indicating a lower bacterial load (McCleary & Henshilwood 2015) of infection in mussels. Generally, CT levels observed indicated that infection levels were low and unlikely to cause mortalities (Marine Institute Pers. Comm.), though some individuals had heavy levels of infection. In this study, OsHV-1 µVar bred oysters were used, it is not sure if these species are also more *V. aestuarianus* resistant, but high likely it could have caused reduced prevalence in *C. gigas*.

A significantly higher prevalence of *V. aestuarianus* was observed within *Mytulis* spp. compared with other marine species *C. maenas* and *C. edule.* After Study 1 was performed in 2015, the Marine Institute suggested that mortalities in *C. gigas* associated with *V. aestuarianus* in Ireland mainly occurred in September and October (Annual NRL meeting on 28-30th of March 2017). In agreement higher

bacterial load of *V. aestuarianus* in mussels was detected during autumn months in Spain (Romero et al. 2010). Therefore, I may have missed the higher prevalence of *V. aestuarianus* in *C. maenas* and *C. edule* as sampling took place from end April till mid-August. *V. aestuarianus* was not detected in *Mytulis spp.* in July in Clonea beach, while a prevalence of 16.7% was observed in September. However, this pattern was not observed for oysters collected at trestles in Dungarvan as both age classes had a higher prevalence at end of June compared with beginning of September.

Previous studies have been succesfully using hydrodynamic models to gain better understanding of the spread of waterborne pathogens in the marine environment (Kough et al. 2015; Foremann et al. 2015). To fully understand the spread of *V. aestuarianus* in the marine environment it would be recommended to develop specific hydrodynamic models including tidal currents, residence time etc. and that take into account environmental factors like pH, salinity and oxygen levels. Although these models demand an extensive amount of data for accurate parametrization, they have proven to fill gaps and gain better understanding of the underlying dynamics (Harvell et al. 2004).

Besides its spread into the environment it would be of interest to investigate the disease development of the bacterium in other marine invertebrate. Investigation the development of infection in other tissues groups by performing histology or by developing an *in situ* hybridization protocol. Laboratory trials could investigate the effect of infection by *V. aestuarianus* on physiological ability or mortalities within invertebrates *C. maenas*, *C. edule* and *Mytulis spp*. and the possibility of disease transmission from these marine species to the host species *C. gigas*. Findings from this study give a preliminary insight into the *V. aestuarianus* pathogen-host-environment interplay. This study once again has shown that a significant oyster pathogen *V. aestuarianus* is able to maintain itself outside its natural host and can extend its geographic range fromthe main hotspot of infection.

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Chapter 5.
Evaluation of the impact of different shore heights on oyster culture, at a disease endemic culture site

Abstract

Pacific oyster *Crassostrea gigas* cultured stocks have suffered significant summer mortalities for the last sixty years worldwide. Past studies have investigated the causes and mechanisms of these mortality events, which have been associated with a complex aetiology involving environmental factors, but have been mainly caused by oyster pathogens such as Ostreid herpesvirus-1 microVar (OsHV-1 Var) and bacteria *Vibrio* spp. As eradication of pathogens and disease is almost impossible in open water systems, management of the disease relies on surveillance, movement restrictions, husbandry techniques and selective breeding programs. In this study, the influence of culturing oysters at different shore heights was investigated by looking at the effects of air exposure on oyster survival, growth and disease development. The study was carried out at an Irish Pacific oyster culture site, where a number of disease events have occurred over the past ten years. Oyster seed and half-grown oysters (approximately 1 years of age) of two different families (Irish naive and French selectively bred to be disease resistant) were held at two shore heights (750 meter and 1200 meter down from shore line) and were monitored over 16 months. Both OsHV-1 µvar and *Vibrio aestuarianus* were found in all treatments. Higher pathogen levels were observed in selectively bred animals, however disease prevalence did not show to be an indicator for performance(growth and survival). The naïve oysters had higher mortality levels compared to the selectively bred group – the latter also performed better in terms of growth. "Resistant" French stock performed better in survival and growth better than "Naïve" Irish stock, Resistant" French stock had better growth at high shore compared to lower shore. The study demonstrated that culture that involves longer exposure times can reduce the presence and impact of pathogens in particular stocks of oysters and can be beneficial in reducing losses particularly at times when potential impacts from pathogens can be greater.

Key words

Crassostrea gigas, Pacific oysters, Performance, Disease development, Herpes virus, *Vibrio aestuarianus*, shore heights, culture techniques,

5.1. Introduction

Infections with parasites, bacteria or viruses in marine shellfish are not rare and can be exacerbated under high density rearing conditions (Bower et al. 1994). High mortality rates in many of the main aquaculture species like Pacific oysters *Crassostrea gigas* have been reported worldwide for decades (Koganezawa 1975; Glude 1975; Maurer & Comps 1986; Clarke 1996) and continue to cause significant economic losses. (FAO 2017b) As a result, improved husbandry techniques that take into consideration management methods for diseases in marine cultured species are a priority in areas that are impacted by heavy losses.

Crassostrea gigas was initially introduced into Europe from Japan in the 1970s due to heavy disease losses in the native oyster *Ostrea edulis* (FAO 2017a; Comp et al. 1980; Grizel & Tige 1982; Cochennec et al. 2000; Beaumont et al. 2002; Culloty et al. 2004) and nowadays it is commercially important worldwide (FAO 2017b). However, since the early 1990s summer mortalities in *C. gigas* have occurred and have been associated with a virus, Ostreid herpesvirus-1 (OsHV-1) (Clarke 1996; Cotter et al. 2010). In 2008 and 2009, a new herpes variant was detected, ostreid herpesvirus-1 microVar (OsHV-1 µVar), which was associated with abnormal *C. gigas* mortality rates (Segarra et al. 2010; Dégremont et al. 2013). In recent years another significant oyster pathogen, bacteria mainly associated with *Vibrio* strains, *Vibrio splendidus* (Garnier et al. 2007; Saulnier et al. 2009; Saulnier et al. 2010) and *Vibrio aestuarianus* (Le Roux et al. 2002; Gay et al. 2004; Saulnier et al. 2010*)* has been detected during *C. gigas* mortality events. However, *C. gigas* summer mortalities are not just the results of pathogens (Arzul et al. 2002; Segarra et al. 2010; Dégremont et al. 2013, Clegg et al. 2013; Travers et al. 2015) but are suspected to be related to a complex aetiology, including interactions between the physical status of the host and environmental parameters (such as temperature, nutrient level and salinity) (Samain and McCombie 2008; Sauvage et al. 2009; European Food Safety Authority 2010; Lynch et al. 2012). Once introduced into a culture area, eradication of these

pathogens and diseases has not been possible to date (Garcia et al. 2011; Pernet et al. 2016), and so potential prevention or control strategies are required.

The susceptibility of oysters to pathogens can differ between age classes; for instance viral strains of ostreid herpes virus-1 variants are found within all oyster age classes, however, mortality due to microvariants mainly affects oysters up to 18 months (Oden et al. 2011; Green et al. 2016), while *V. aestuarianus* has been reported to be associated with adults (Travers et al. 2015; Green et al. 2016). A study by Petton et al. (2015) showed the complexity of disease expression within *C. gigas*, by demonstrating that replication of viral strains only induced *C. gigas* mortalities after the introduction of *Vibrio* spp. (Petton et al. 2015).

Selective breeding programs in France were set up to produce and culture a new cohort of *C. gigas* with higher resistance to OsHV-1 µVar (Dégremont 2011). Subsequent studies highlighted that herpes virus resistance was a highly heritable trait in *C. gigas* (Dégremont 2015a; Dégremont 2015b) resulting in lower summer mortality rates in all age classes compared to counterparts from the wild (Dégremont 2011; Dégremont et al. 2015a). This suggested that susceptibility of oyster pathogens and diseases not only differs between age classes but also between genetically different families. To the best of our knowledge, a selective breeding program focussing on *V. aestuarianus* resistant oysters has not been described in the literature to date. However, a recent study showed higher resistance of *V. aestuarianus* in juveniles of OsHV-1 resistant cultured families, suggesting dual resistance in juveniles, while this pattern was not observed for adults (Azéma et al. 2015).

Besides breeding programs, previous studies have assumed that aquaculture farming practices, like ropes and basket, might play a role in disease and pathogen development (Garcia et al. 2011; Pernet et al. 2012; Pernet et al. 2014). Within the Irish *C. gigas* industry, the main culture methodology involves the holding of oysters in bags on trestles in intertidal areas and a small percentage is cultured by bottom culture (Heffernan 1999; European Food Safety Authority 2010). Natural oyster reefs have been shown to have a higher density of marine organisms compared to areas without oyster reefs (Hosack 2003; Escapa et al. 2004;

Dumbauld et al. 2009). Subsequently, holding oysters in the water column above the sediment reduces the risk of predation found at the sediment layer (Oesterling & Petrone 2012; Walton et al. 2013). Moreover, holding the oysters intertidally gives the oyster farmers the opportunity to easily access and control their stock during low tide and reduce handling costs (Gittings & O'Donoghue 2012). However, longer aerial exposure reduces feeding time, resulting in reduced growth rates, introduces stress, resulting in an unfavourable energetic balance, thus increasing susceptibility to pathogen and diseases resulting in oyster mortalities (Oesterling & Petrone 2012). However, air exposure may also be beneficial in that it might control biofouling of hydroids, bryozoans, sea squirts, etc. on the oyster bags, which compete with the oysters for food and oxygen (Oesterling & Petrone 2012). Also, as OsHV-1 is waterborne, with horizontal transmission of the virus through the water column (Sauvage et al. 2009; Schikorski et al. 2011), it has been suggested that less immersion time will lead to a lower exposure time to viral compounds (EFSA 2015). Indeed, previous research showed that lowering immersion time could have a significant effect on mortalities and even reduce mortalities by up to 50% (Soletchnik et al. 2011; Schikorski et al. 2011; Paul-Pont et al. 2013; Whittington et al. 2015). Therefore, exposure/immersion time is a factor affecting performance and disease development in *C. gigas* – the extent to which is unclear.

A study that investigated the most suitable conditions for recruitment and *C. gigas* performance (growth and survival) in artificial oyster reefs along different exposure gradients ranging from 0 to 70% exposure time, found that shell length, condition index, ash free dry weight and internal cavity volume for both recruits and adults were negatively correlated with tidal emersion (exposure time) (Walles et al. 2016). However, optimum exposure time to balance positive and negative aspects was found to be correlated with age of oysters (Walles et al. 2016). Although the construction of artificial oyster reefs is not comparable to the oyster culture method employed in Ireland, it still shows that there is an effect of tidal emersion on oyster performance, which might differ between age classes.

Mortalities associated with oyster pathogens can vary between oyster age classes (Arzul et al. 2002; Renault 2006; Oden et al. 2011) and oyster genetics (Dégremont

2011; Dégremont et al. 2015b). Disease expression has been reported to be a result of a complex interaction between Ostreid herpes virus -1 variants and *Vibrio* species (Petton et al. 2015) and can differ between culture methods (Garcia et al. 2011; Pernet et al. 2012; Pernet et al. 2014; Petton et al. 2015). To the best of our knowledge the effect of air exposure and shore height on disease development in different age classes and families (naïve and selectively bred disease resistant) of *C. gigas* have never been investigated.

In this study, the effects of shore height (difference in air exposure) on disease development and performance, i.e. growth and mortality, of two age classes of two families of *C. gigas* with varying pathogen susceptibility was investigated to try to optimise husbandry techniques in the presence of pathogenic organisms. A field trial was performed at one of the main Irish *C. gigas* culture sites, with a history of OsHV-1 µVar and *V. aestuarianus*, from June 2016 up to September 2017. Different age classes and families were held at different shore heights with 6-8 hours difference in air exposure to determine overall impact on growth and survival.

5.2. Material and Methods

(1) Study sites

The field trial took place at an Irish oyster culture site; Dungarvan (Figure 1), Co. Waterford (52.0936 °N -7.6204°W), which has a history of OsHV-1 µVar (www[1]) and *Vibrio aestuarianus* (Pers. Comm. with Marine Institute). The oyster culture site in Dungarvan is sheltered, being almost closed off by the linear Cunnigar spit to the east (www[2]). Intertidal habitats are dominated by sandflats and it has mudflats at the edge of saltmarsh habitats. The water quality of Dungarvan Harbour varies from moderate to good, indicating unpolluted water and acceptable levels of biochemical oxygen demand (EPA 2015). Changes to height of trestle and rotation of trestles bags did not show significant changes in yield, suggesting that the production is not currently food-limited (UISCE project report material BIM. 2007).



Figure 1. Pacific oyster culture site in Dungarvan

(2) Field trial

In Dungarvan when oysters reach a minimum of 4 mm size, they are transported from the nursery into bags and placed at the top of the culture site approximately 750 meter from the top shore line (denoted as high shore) – from which the second group of oysters were moved approximately 500 meter down (1200 meter from the top shore line and denoted as low shore). In the first year they are turned over 3 times, to promote best growing and performance. Over the next couple of years oysters are graded, reduced in densities and transported to bags with larger mesh sizes and moved down to the low shore – where our oysters were placed. Just before reaching consumption size, oysters are transported to harvestable areas which are spread out over the whole farm. When reaching consumption size, at 3 years of age, the oysters are brought in, packaged and sold (Oyster farmers Pers. Comm; UISCE project report material BIM 2007).

The two different families used in this study were (a) Irish produced "naïve" seed and half grown *C. gigas* cultured at an oyster hatchery in Galway Bay from subtidal broodstock (53°09′16.27′′N, 9°04′58.19′′W) (Table 1), which have never been exposed to pathogens like OsHV-1 µVar and *Vibrio aestuarianus* – denoted "Naïve" Irish for this study and (b) seed and half grown *C. gigas* imported from France hatcheries, which have been selectively bred to be OsHV-1 µVar resistant - denoted as "Resistant" French for this study (Table 1).

Table 1. Average weight (gram) and length (mm) ± standard deviation of oysters from both age groups and families when measured prior to the trial commencing (initial sample at T (0))

	Average weight	**Weight range**	**Average length**	**Length range**
"Resistant" French seed	0.5 ± 0.1	0.2 – 1.1	12.2 ± 2.3	7.2 – 18.6
"Naïve" Irish seed	0.5 ± 0.2	0.3 – 1.2	15.0 ± 2.5	10.1 – 20.8
"Resistant" French half grown	16.0 ± 6.0	3.5 – 31.1	63.0 ± 9.8	44.4 – 85.2
"Naïve" Irish half grown	8.8 ± 5.7	2.4 – 31.2	52.5 ± 10.7	29.4 – 80.2

On the 2nd of June 2016, oysters of different age classes and different families were relayed to the oyster culture site at the two shore heights in Dungarvan. Density of the oysters in the bags and bag mesh size were similar to those used by the oyster farmers for each oyster age group. Group one, which consisted of a cohort of both oyster families and age groups, were held in bags (Table 2) on trestles at the highest shore height of the farm (750 meters from shore line), which is always accessible during low tides and Group two, a cohort of both oyster families and age groups, was held in oyster bags on trestles at the lowest shore height of the culture site (1200 meters from shore line) (Figure 2), which was only accessible at spring tide, approximately 6-8 hours difference in air exposure.

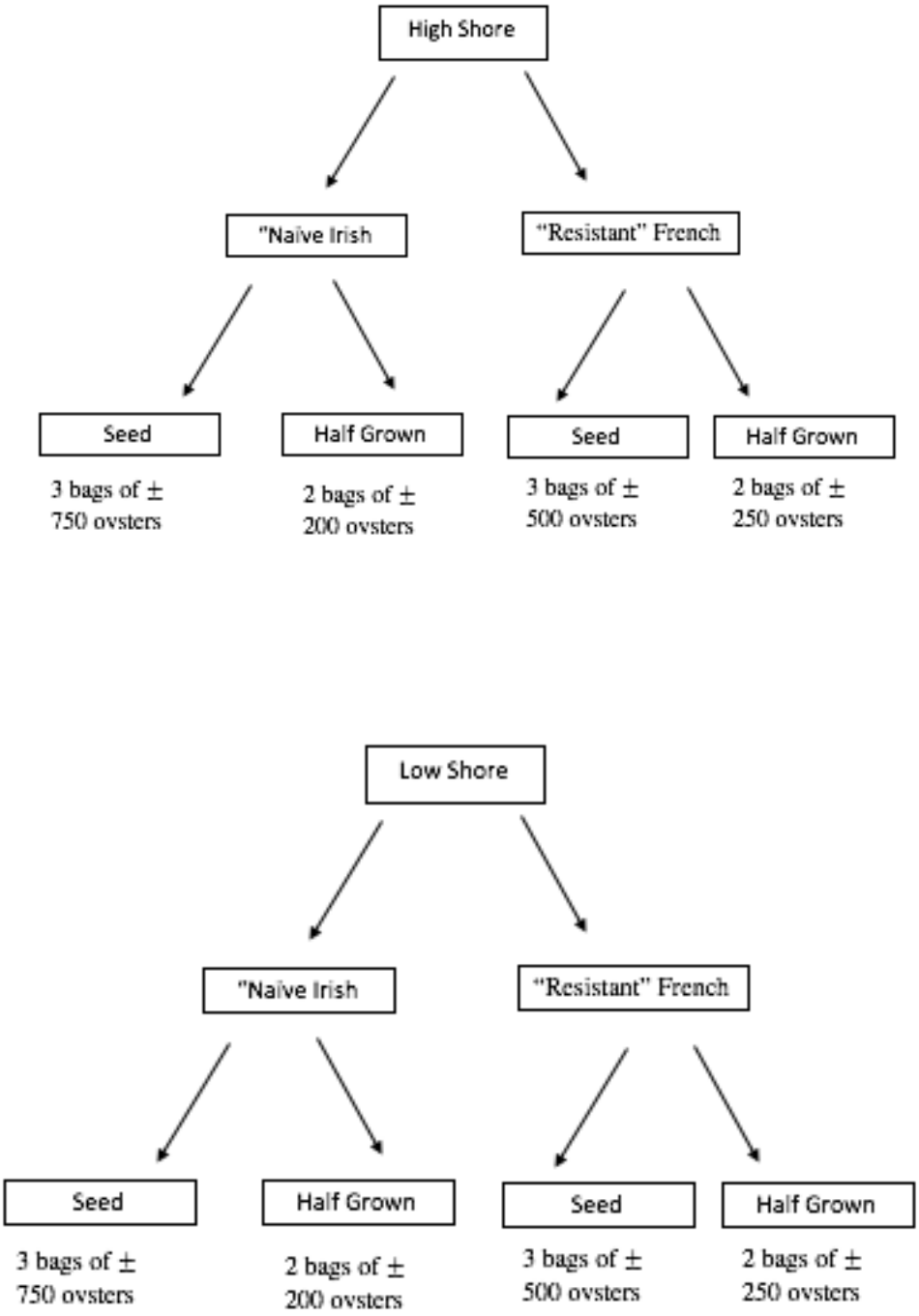


Figure 2. Number of oysters relayed per age class and family at high and low shore

An initial sample of 30 *C. gigas* per family and age group was taken the same day the oysters were relayed to the shore. It was decided to monitor the oysters' progress one year after the trial commenced to give the oysters time to grow and to review the effects of a growing season on performance at the different shore

heights. The oysters were sampled for the first time on the 23rd June 2017 (56 weeks post relaying). The oysters were sampled again on the 6th September 2017 (68 weeks post relaying) to gain a better understanding of specific effects of the summer months on survival and disease prevalence.

One year after the field trial commenced, two bags of the "Resistant" French seed at the high shore were washed away and were not detected elsewhere at the oyster culture site and so only one bag of this seed was available for screening during the summer of 2017. Also, both age classes of "Resistant" French stock at the low shore site had gone missing. As the farmer was culturing these same oysters at the same lower shore height and they had originally been relayed in Dungarvan at the same time as our experimental oysters a new cohort of the "Resistant" French oysters were sampled from the oyster farmers stock so that oyster performance and disease development of "Resistant" French stock at the lower shore could be investigated in June 2017 and September 2017 as for the experimental groups. We used our previous initial sample as a starting datafor this group.

(3) Environmental parameters

Environmental (salinity, pH and temperature) data loggers (Star-Oddi) *in situ* at the oyster trestles were used to measure and record water temperature continuously every hour from middle of May until the end of September 2017. Average water temperatures were calculated as average temperature per day for the time submerged.

(4) Mortality, cumulative mortality and oyster sampling

Percentage mortality was estimated by counting the first 100 oysters per bag by counting coupled empty shells. After removal of all dead oysters, remaining live oysters were placed back in the bags. Survival rates corresponded to the number of live individuals when counting the first 100 oysters. Cumulative mortality was also calculated based on percentage live oysters available in the two sampling points in June and Sept 2017.

After the mortality count was carried out on each bag, a subsample of 30 live oysters per age class, per family and at both shore heights were collected and

transported to the laboratory for further processing and screening. In the laboratory, the gill tissue (5mm^2) of *C. gigas* was frozen at -20°C for DNA isolation.

(5) Molecular diagnostic screening

DNA extraction

Before DNA extraction took place, tissues were washed in double deionized water (ddH_2O) and blot dried using tissue paper. DNA extraction was performed using the Chelex-100 methodology (Walsh et al. 1991). Tissue samples (approx. 5mm^2) were placed in a 10% chelex solution (100 microliters volume) (Sigma Aldrich) and following the samples were placed in a Hybaid thermal cycler for 1 hour and 10 minutes heated at 99ºC to facilitate cell lysis (Walsh et al. 1991). A subsample of the DNA was quantified by a spectrophotometer (NanoDrop 1000 spectrophotometer) to confirm DNA quantity and quality.

Polymerase chain reaction (PCR) screening for ostreid herpesvirus-1 microVar

Standard PCR to detect OsHV-1 µVar was performed using the OHVA/OHVB primers (Lynch et al. 2013). Positive controls (duplicate) consisting of OsHV-1 µVar infected oyster tissue and negative controls (duplicate) of double distilled water (ddH_2O) were used for each PCR. Agarose gel electrophoresis was carried out using a 2% agarose gel stained with ethidium bromide (10mg/l stock) and was run with an electrical charge of 110V for 45-60 minutes. Expected size of amplified PCR products for OsHV-1 µVar was 385 bp (Lynch et al. 2013).

Quantitative PCR (qPCR) for Vibrio aestuarianus

qPCR was performed for the detection of *Vibrio aestuarianus* following the protocol of McCleary and Henshilwood (2015). A total of 5 µl of DNA per oyster was used to detect *V. aestuarianus* prevalence. The master mix for qPCR existed of a total volume of 20 µl; 5.62 µl dH_2O, 0.75 µl of 5 *µM* forward primer (dnaJ f420), 0.75 µl of 5 *µM* reverse primer *(dnaJ r456))*, 0.38 µl of 5 *µM* of probe (*dnaJ* p441) and 12.5 µl of 2x Taqman mastermix. All samples were performed in duplicate. Negative controls (n=2) of 5 µl double distilled water (ddH_2O) were used. Positives controls (n=2) contained 5 µl *V. aestuarianus* infected DNA. A tested sample is considered positive if its mean CT value is below 37.

(6) Statistical analyses

Statistical analyses were performed in Statistical model program R studio (R core team 2013). Normality was tested using the Shapiro-Wilks Normality test. Length and weight data was analysed following student t tests. Pearsons Chi-squared tests were used to test prevalence of infection. For all analyses, a critical value of 0.05 was used to confirm significant results. Data are presented as mean ± standard deviation.

5.3. Results

Environmental parameters

Summer 2016 indicated a moderate temperature, with days reaching water temperature of 16 °C in July and August. In June 2017, the first two weeks were wet and dull, introducing dry weather with warm temperatures and lot of sunshine from mid-June up till the end of June. July was more variable with colder temperature and rain and showers most days. August showed a similar pattern as July with lower temperatures and more rain as usual for the month (http://www.met.ie/climate/monthly-weather-reports.asp).

Average water temperatures higher than 16 °C were observed from the 17th of June 2017, followed by 7 warm days, before the oysters were sampled on the 23rd of June. Average water temperatures during summer remained below 16 °C. However, just a few days before sampling on the 6th of September 2017, temperatures just dropped below 16 °C (Figure 3).

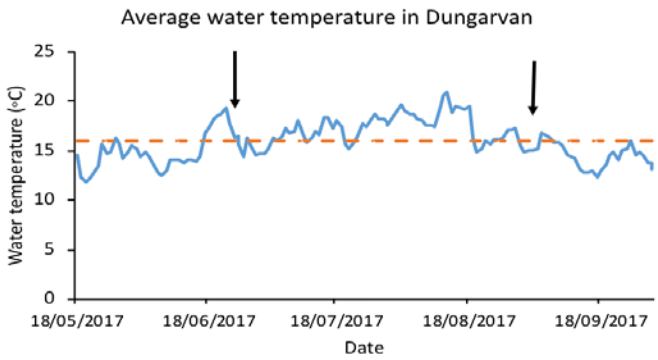


Figure 3. Average water temperature for oyster farm in Dungarvan from mid of May till end of September 2017 (Arrows indicating sample dates)

Mortality and cumulative mortality

"Naïve" Irish seed had a very high mortality rate of 70% 56 weeks post relaying and when further sampling took place 68 weeks post relaying the remaining oysters indicated a mortality of 97%. 56 weeks post relaying mortalities were significantly higher in the seed age group compared to their older counterparts. At the high shore "Naïve" Irish seed showed a 70% mortality while half grown only indicated a 3% mortality at the high shore ($X^2 = 26.46$, df = 1, $P < 0.01$). At lower shore "Naïve" Irish seed showed a 22.7% mortality while half grown indicated an 8% mortality at the lower shore ($X^2 = 8.31$, df = 1, $P < 0.01$). "Resistant" French seed indicated a mortality of 12.3% in contrast to a 1.5% mortality for the half grown at the high shore ($X^2 = 9.01$, df = 1, $P < 0.01$). At lower shore and Resistant" French seed showed a 12% mortality while half grown indicated an 2% mortality at the lower shore ($X^2 = 7.68$, df = 1, $P < 0.01$).

68 weeks post relaying, not much "Irish Naïve" seed was left in the bag as there was already high mortality observed 56 weeks post relaying. No differences in mortality rates were observed for "Naïve" Irish half grown (12%) compared to their younger (7%) counteracts at high shore ($X^2 = 1.45$, df = 1, $P = 0.228$), again no differences in mortality were observed at lower shore with mortalities of 14% for both half grown and seed ($X^2 = 0$, df = 1, $P = 1$). At high shore, "Resistant" French half grown indicated a lower mortality with 7% compared to the "Naïve" Irish half grown (15.5%), but not significantly ($X^2 = 0$, df = 1, $P = 0.057$). (Figure 4).

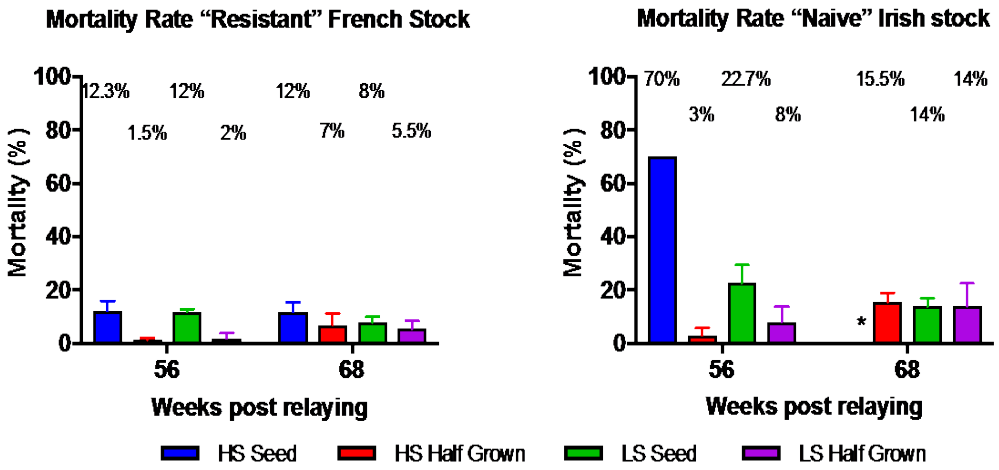


*Figure 4. Mortality rate of "Resistant" French stock (left) and "Naïve" Irish stock (right) for 56 and 68 weeks post relaying at two shore heights (HS = High Shore, LS = Lower Shore) during the summer of 2017 / * No mortality shown for "Naïve" Irish seed at high shore 68 weeks post relaying because less than 100 oysters left*

"Resistant" French seed indicated a significantly higher (X^2 = 18.71, df = 1, P < 0.01) cumulative mortality at high shore (23%) compared with lower shore (16.7%). Also, for half grown "Resistant" French showed higher cumulative mortality, but not significantly (X^2 = 3.01, df = 1, P = 0.08) at high shore (8.4%) compared with the lower shore (5.6%)**.** "Naïve" Irish seed indicated a significantly higher cumulative mortality (X^2 = 2199.75, df = 1, P < 0.01) at high shore (99.5%) compared with the lower shore (33.5%). Again, also half grown "Naïve" Irish oysters did not indicate a significant difference in mortality (X^2 = 2.08, df = 1, P = 0.15) between high shore (17%) and lower shore (21%) (Figure 5).

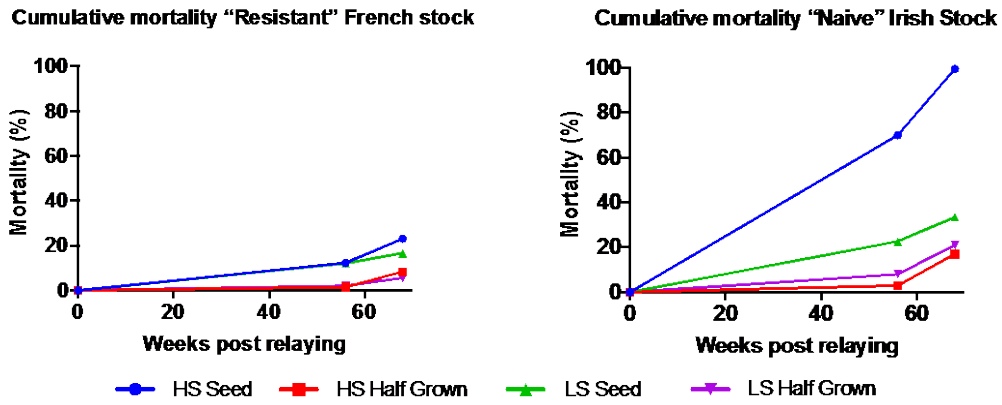


Figure 5. Cumulative mortality rate of French stock (left) and Irish naïve stock (right) for 56 and 68 weeks post relaying at two shore heights (HS = High Shore, LS = Lower Shore) during the summer of 2017

Growth

The initial sample showed a similar mean whole wet weight for both the "Naïve" Irish (0.5± 0.2 gram) and "Resistant" French seed (0.5± 0.1 gram) ($t = 0.48$, $df = 58$, $P = 0.64$), while the mean length was significantly larger ($t = 4.45$, $df = 58$, $P < 0.01$) for "Naïve" Irish seed (15.0 ± 2.5 mm) compared to the "Resistant" French seed (12.2 ± 2.3 mm). The "Resistant" French seed and half grown oysters both had highest growth rates over the study period and both groups performed better at the high shore site. The "Resistant" French half grown were significantly heavier ($t = 5.00$, $df = 58$, $P < 0.01$) (16.0 ± 6.0 compared with 8.8 ± 5.7 gram) and bigger ($t = 4.34$, $df = 58$, $P < 0.01$) (63.0 ± 9.8 compared with 52.5 ± 10.7 mm) to the "Naïve" Irish half-grown oysters.

"Resistant" French stocks seemed to have higher growth rates compared with "Naïve" Irish stock (Figure 6 and Figure 7). Overall growth patterns indicated that "Resistant" French half grown oysters performed significantly better at higher shore than lower shore (56 weeks: weight $t = 4.41$, $df = 56$, $P < 0.01$ & length $t = 3.76$, $df = 56$, $P < 0.01$; 68 weeks: weight $t = 2.78$, $df = 58$, $P < 0.01$ & length $t = 1.37$, $df = 58$, $P = 0.17$). Similar patterns were observed for "Resistant" French seed but those differences were not significant (56 weeks: weight $t = 1.82$, $df = 58$, $P = 0.07$ & length $t = 2.29$, $df = 58$, $P = 0.03$; 68 weeks: weight $t = 1.04$, $df = 58$, $P = 0.3$ & length $t = 1.04$, $df = 58$, $P = 0.3$). No differences in growth patterns were observed for "Naïve" Irish half grown within the first 56 weeks, however after 68

weeks oysters performed better lower shore (56 weeks: weight t = 0.21, df = 58, P = 0.83 & length t = 0.97, df = 58, P = 0.33; 68 weeks: weight t = =2.52, df = 58, P = 0.01 & length t = 3.38 , df = 58, P < 0.01). A similar pattern was observed for the “Naïve” Irish seed, however these analyses were affected by low survival at lower shore (56 weeks: weight t = 5.20, df = 31, P < 0.01 & length t= 6.63, df = 58, P < 0.01; 68 weeks: weight t = 2.48, df = 31, P = 0.02 & length t=4.25, df = 31, P < 0.01).

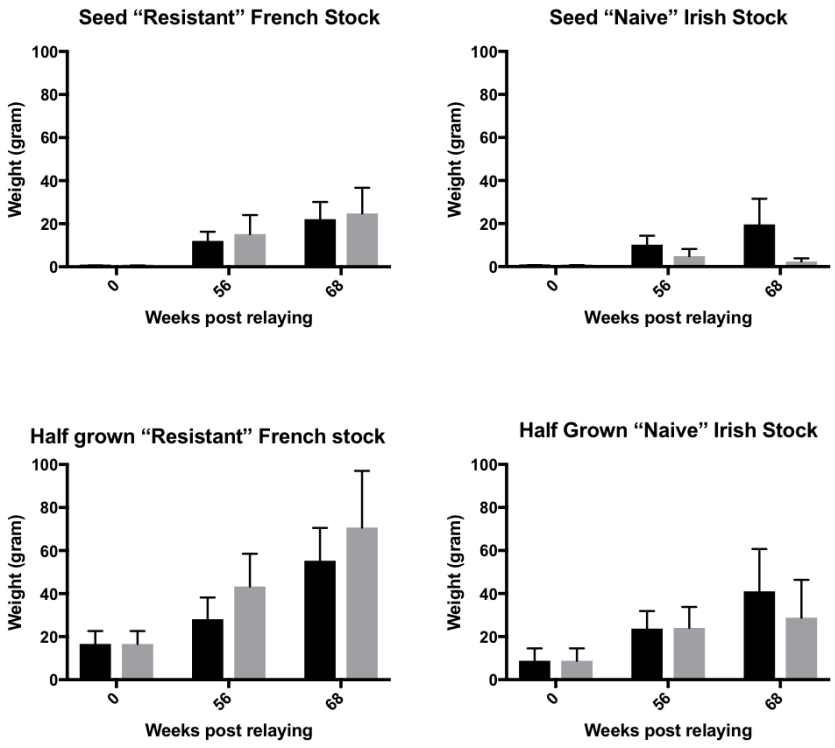
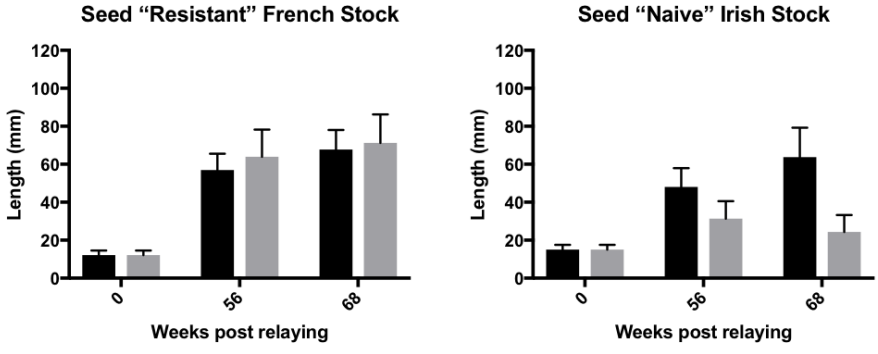


Figure 6. Mean weight (g) per oyster family and age class for 56 and 68 weeks post relaying at low shore (black bar) and high shore (grey bar)



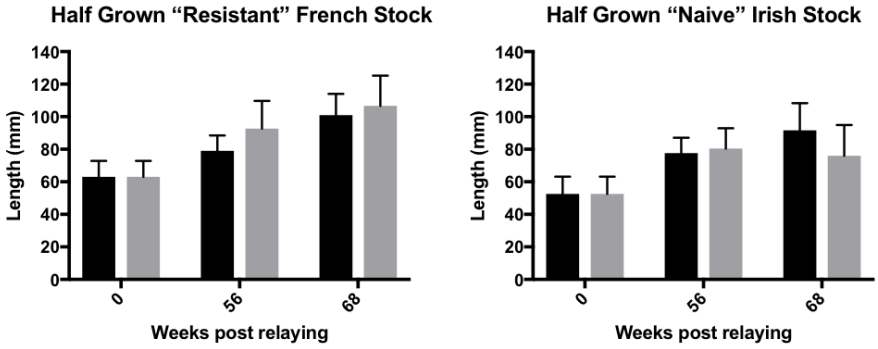


Figure 7. Mean length (mm) per oyster family and age class for 56 and 68 weeks post relaying at low shore (black bar) and high shore (grey bar)

Infection of OsHV-1 µVar

In the initial sample taken at the day of relaying (2-6-2016) no OsHV-1 µVar was detected in the seed from both "Naïve" Irish stock and "Resistant" French stock and in the half grown "Naïve" Irish stock. Only "Resistant" French half grown had a prevalence of infection of 6.7% (n = 2/30).

56 weeks post relaying (23-6-2017) prevalence of OsHV-1 µVar was observed in all age classes and families of Pacific oysters at high shore and low shore. At high shore, lowest OsHV-1 µVar prevalence was observed for the "Resistant" French seed (6.7%) and highest prevalence for "Naïve" Irish half grown (40%). At the low shore, the "Naïve" Irish seed showed the lowest prevalence of OsHV-1 µVar (6.7%), while "Resistant" French seed had the highest prevalence (73.3%). Both age classes of the "Resistant" French stock obtained from the farmers main stock

at the lower shore had a high prevalence of OsHV-1 μVar at 73.3% in seed and 46.7% in half-grown oysters.

Very little infection was observed at 68 weeks post relaying in Sept 2017 with only "Naïve" Irish half grown at the high shore site (3.3%) and" Resistant" French half grown at the low shore (43.3%) demonstrating the presence of the virus (Figure 8).

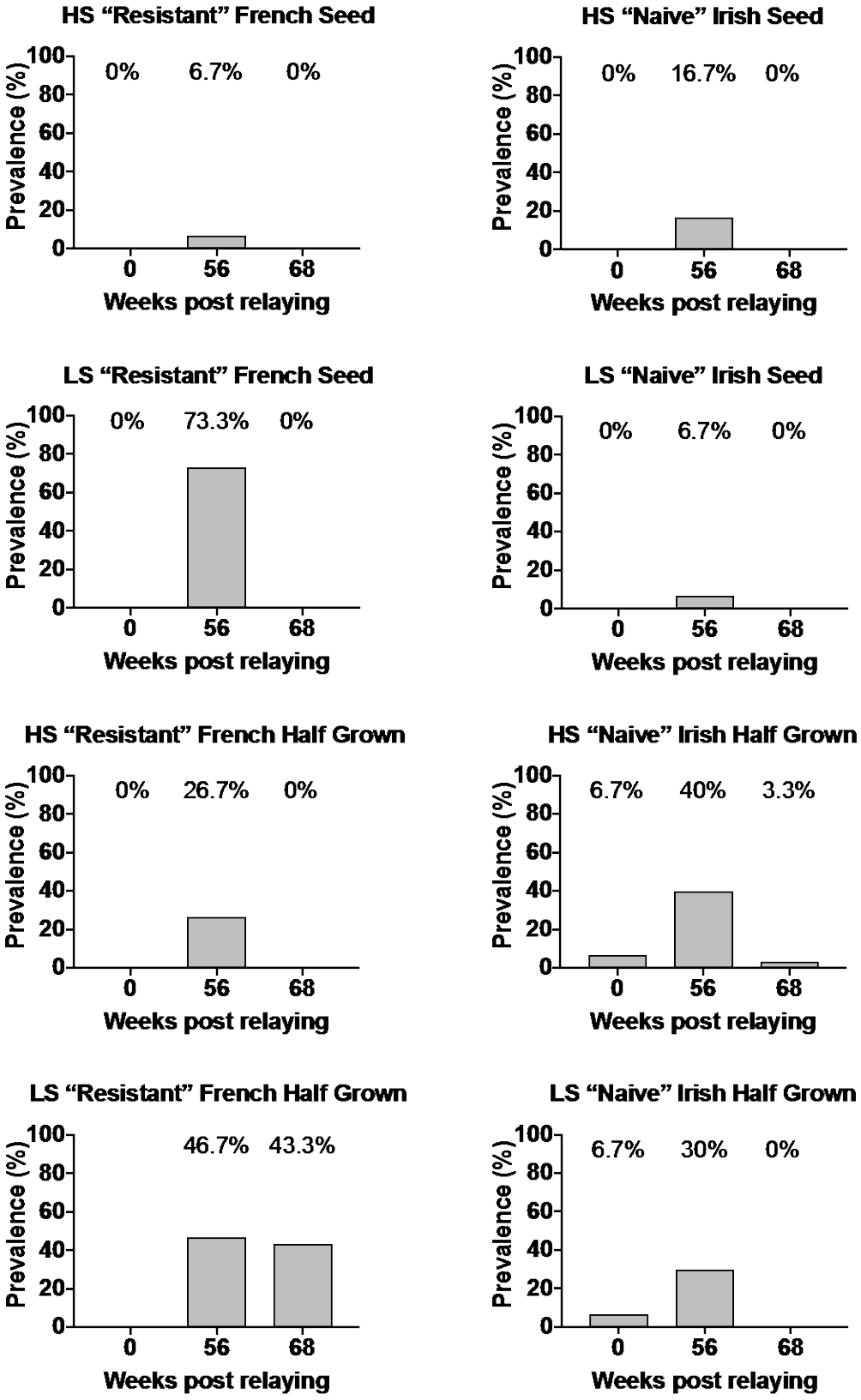


Figure 8. Prevalence of OsHV-1 µVar per family and age class of French stock and Irish naïve stock for 56 and 68 weeks post relaying at two shore heights (HS = High Shore, LS = Lower Shore) during the summer of 2017 following initial relaying to the site in June 2016

Infection of Vibrio aestuarianus

In the initial sample taken at the day of relaying (2-6-2016), *Vibrio aestuarianus* was detected in the "Resistant" French seed (10%) and Irish naïve half grown (16.7%) but not in the Irish naive seed nor the French half-grown.

56 weeks post relaying lowest *V. aestuarianus* prevalence was observed for "Naïve" Irish seed at both high shore and lower shore sites (both 6.7%), while high prevalence of *V. aestuarianus* was observed for "Resistant" French seed (86.7%) and half-grown (73.3%) at high shore and "Naïve" Irish half grown (93.3%) at lower shore. Overall, half-grown oysters of each family had higher prevalence of *V. aestuarianus* than their seed.

68 weeks post relaying the overall pattern of prevalence of *V. aestuarianus* indicated a lower prevalence compared with 56 weeks post relaying except for both seed families at lower shore. *V. aestuarianus* showed a higher prevalence at high shore families and age classes 56 weeks post relaying, while it was more prevalent in lower shore families 68 weeks post relaying (Figure 9).

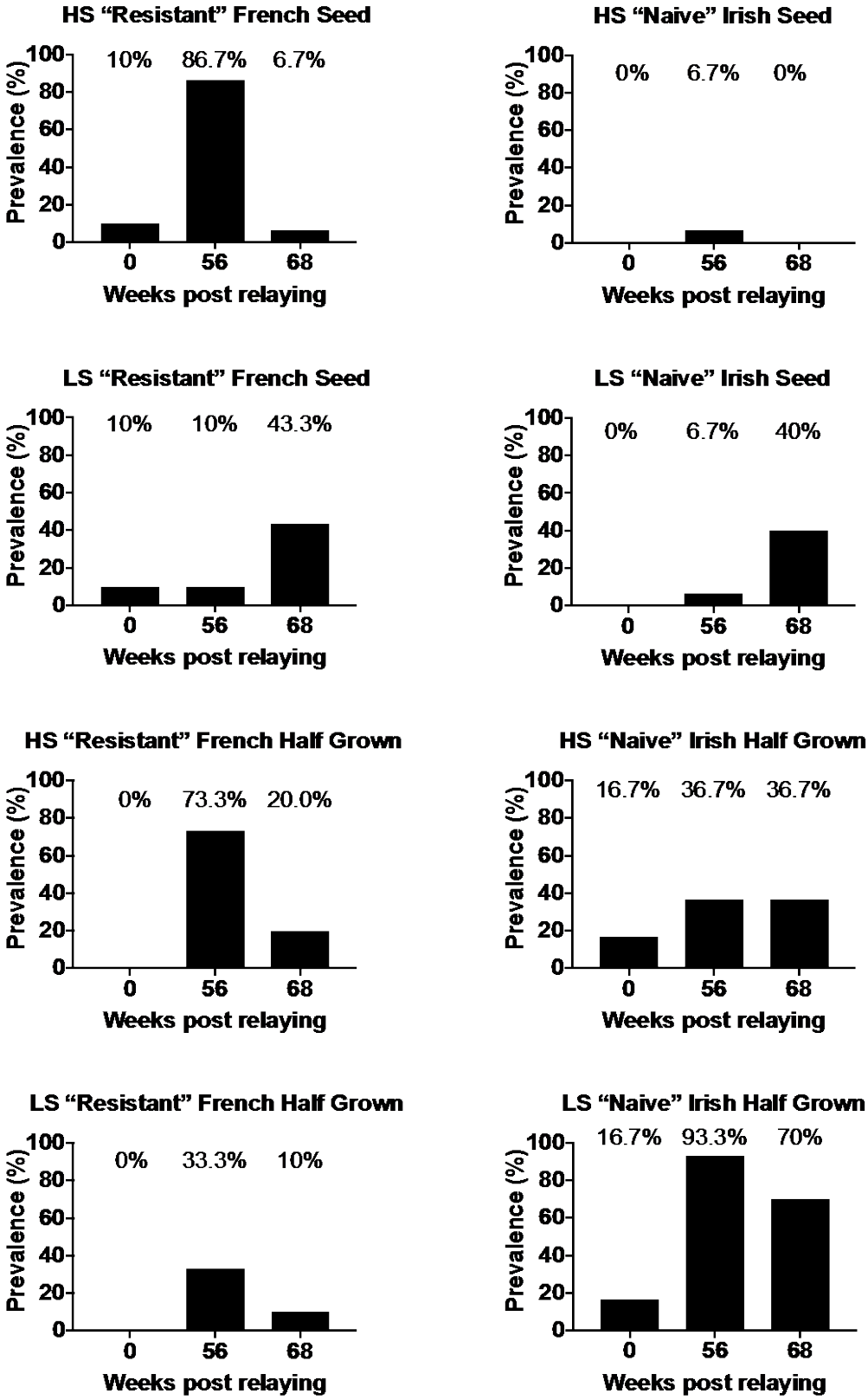


Figure 9. Prevalence of Vibrio aestuarianus per family and age class of French stock and Irish naïve stock for 56 and 68 weeks post relaying at two shore heights (HS = High Shore, LS = Lower Shore) during the summer of 2017

For both sample dates, lower CT values of *V. aestuarianus*, indicating higher infection, was observed in "Resistant" French stock compared with "Naïve" Irish stock (Table 2).

Table 2. Mean and range CT values of Vibrio aestuarianus 56 weeks post relaying

	High Shore Mean/ Range CT Value	**Low Shore Mean/ Range CT values**
56 weeks post relaying (23-6-2017)		
"Resistant" French seed	33.3 / 19.9 – 36.2	35.4 / 35.3 - 35.4*
"Naïve" Irish seed (n=3)	35.7 / 35.3 – 36.0	35.9 / 35.4 - 36.2
"Resistant" French half grown	31.5 / 23.6 – 36.9	35.3 / 27.4 - 36.9*
"Naïve" Irish half grown	36.3 / 34.5 – 36.8	34.3 / 32.5 - 36.3
68 weeks post relaying (6-9-2017)		
"Resistant" French seed	31.2 / 25.4 - 36.9	35.7 / 32.7 – 36.9*
"Naïve" Irish seed (n=3)	-	35.1 / 33.4 – 36.9
"Resistant" French half grown	32.8 / 25.1 – 36.6	32.5 / 27.0 – 36.1*
"Naïve" Irish half grown	35.6 / 33.4 – 36.7	35.3 / 30.9 – 36.5

Co-infection of OsHV-1 µVar and Vibrio aestuarianus

In the initial sample there was no co-infection observed (therefore no data presented at week 0 in Figure 10). However, co-infection of OsHV-1 µVar and *V. aestuarianus* was observed during the field trial in all age classes, families and shore heights with the exception of "Naïve" Irish seed at high and lower shore.

56 weeks post relaying "Naïve" Irish half-grown (20%) indicated significant higher ($X^2 = 6.67$, df = 1, $P < 0.01$) prevalence of co-infection than their seed (0%) at high shore. Again Naïve" Irish half grown (26.7%) had significant higher ($X^2 = 9.23$, df = 1, $P < 0.01$) co-infection than their seed (0%). This pattern was also observed for "Resistant" French stock at high shore ("Resistant" French half grown 10% / "Resistant" French seed 6.7%), but not significantly ($X^2 = 0.22$, df = 1, $P = 0.64$). "Resistant" French seed (80%) and half-grown (60%) showed higher prevalence of *V. aestuarianus* than naïve seed Irish oysters (6.7%) and half-grown

(16.7%) at high shore. At both low and high shore, “Naïve” Irish stock did not show any co infection.

68 weeks post relaying only one individual of the “Naïve” Irish half grown indicated co infection of OsHV-1 µVar and *V. aestuarianus* at high shore. All the other individuals only showed prevalence of *V. aestuarianus*. There was no OsHV-1 µVar prevalence detected and therefore no co-infection observed at the low shore (Figure 10).

Overall the pattern indicated that single infections of OsHV-1 µVar only not very common. OsHV-1 µVar infection was mainly observed when individuals were also infected with *V. aestuarianus*, while *V. aestuarianus* was often observed as single infections in oysters.

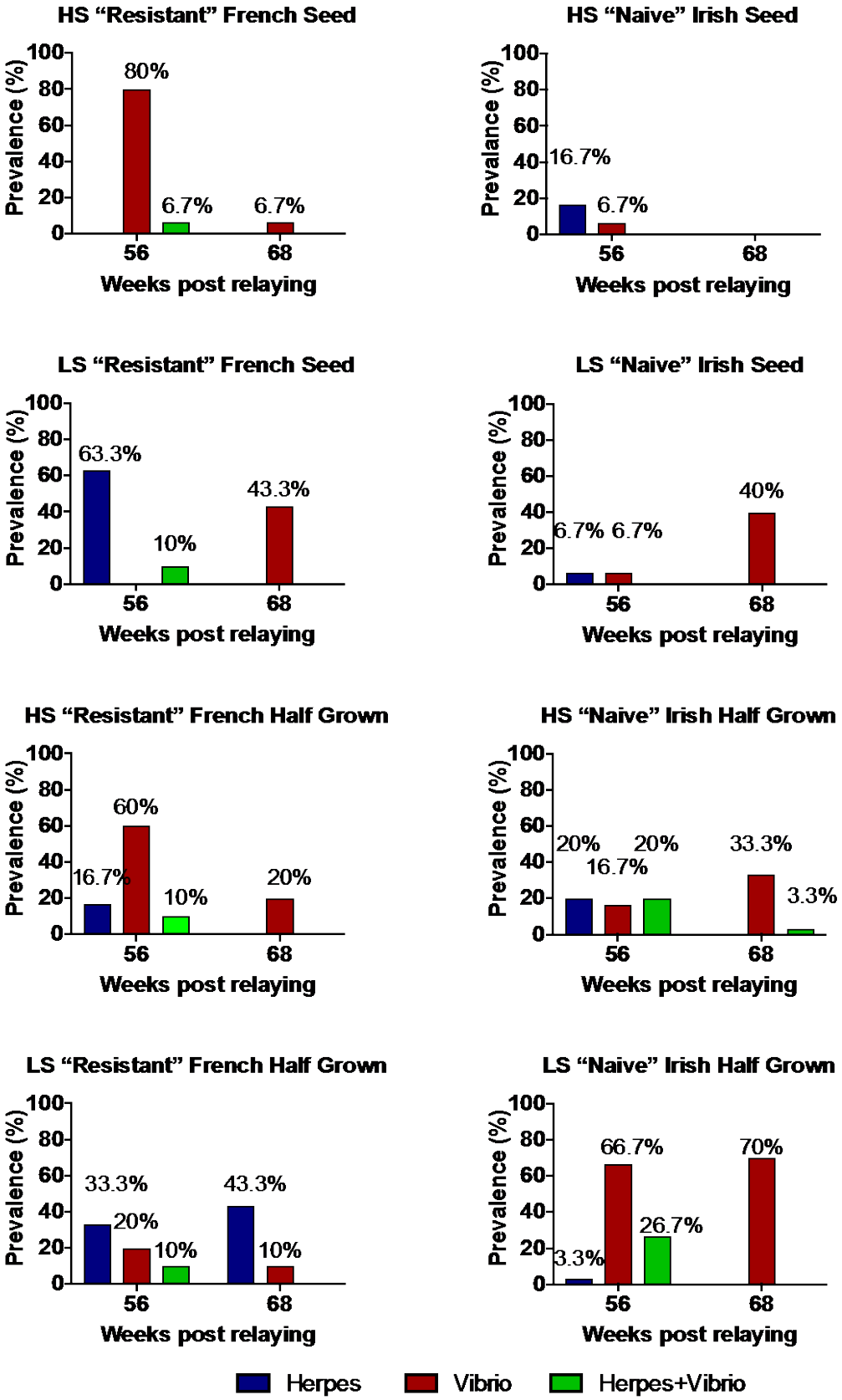


Figure 10. Prevalence of pathogens per individual per family and age class for high shore (HS) and low shore (LS) at 56 and 68 weeks post relaying

5.4. Discussion

The findings of this study indicate, for the first time, that culturing at different shore heights and subsequent air exposure and emersion times can have an impact on *C. gigas* performance and pathogen development (OsHV-1 µVar, *Vibrio aestuarianus* and co-infection) in "Naïve" Irish and selectively bred "Resistant" French families and in different age classes of such families.

Previous studies observed better performance (growth and survival) in oysters that had survived mass mortalities related to diseases (Dégremont et al. 2010a) or selectively bred disease resistant oysters (Dégremont et al. 2010b; Dégremont et al. 2011) compared to naïve oysters. In agreement with these studies, the "Resistant" French oysters performed better overall at both shore heights in terms of growth and survival, even in the presence of pathogens. Findings from this study would indicate that culturing at high shore with shorter immersion time, particularly during the months when pathogens were present, lead to better survival and growth rates for selectively bred animals.

The pathogens present at this site can result in significant rapid oyster mortalities. While the oysters generally had survived well over the first winter, with "Naïve" Irish seed oysters having the highest mortality rate, oysters were much more stressed over the subsequent summer of 2017 when conditions of higher temperatures and pathogen presence prevailed. This resulted in the "Naïve" Irish oysters suffering much more significant losses than the "Resistant" French animals. Those "Naïve" Irish oysters were orginated from a hatchery and therefore adapted to subtidal conditions what might have increased the stress levels at the high shore (conditions with long air exposure). "Resistant" French oysters had very low mortality rates being slightly higher at low shore level. In past studies, oyster mortality due to virus microvariants has been predominantly associated with spat and juveniles (<18 months) (European Food Safety Authority 2010). It has been demonstrated that a high viral quantity induces oyster mortality, however mortalities can be observed in individuals without any viral load, suggesting that the virus is not exclusively inducing oyster mortalities and other pathogens, such as *Vibrio spp.*, may be involved (Petton et al. 2015; Azéma et al. 2016). *V. aestuarianus* has also been linked with mortality events in wild and cultured *C.*

gigas (Le Roux et al. 2002; Gay et al. 2004; Saulnier et al. 2010) and is able to induce mortalities in the absence of OsHV-1 µvar (Petton et al. 2015). As both oyster pathogens OsHV-1 µVar and *V. aestuarianus* were present in this study co-infections were detected, it is likely that a complex aetiology caused any observed mortalities.

The "Naïve" Irish seed experienced significantly higher mortalities at the high shore compared with their cohort at the low shore thus indicating this seed stock from subtidal broodstock is vulnerable to longer air exposure, potentially due to their size and thinner shells and less capacity to stay out of water for longer periods without desiccation occurring – particularly during summer months. At high shore, seed is exposed to higher air temperatures for a longer period, which is introducing another stressor. In addition, previous studies indicated that younger oysters go through a negative energy balance during spring and therefore are more susceptible for diseases. Larger individuals normally have larger energy reserves, and therefore are more likely to be able to fight infections (Pernet et al. 2012). This missing pattern for "Resistant" French seed could be explained, again, by it's selective breeding program.

Growth rates were higher in "Resistant" French oysters. Previous studies showed that oysters grow better (and show lower mortalities) at sites with higher diatom relative abundances, weaker haline and thermal stratification, and smaller temperature variations (King et al. 2006; Cassis et al. 2011). Therefore, higher growth rates at the low shore (longer immersion time) might be expected. In our study, this was most obvious over the summer months in "Naïve" Irish seed.

In contrast, both age classes of the "Resistant" French showed better growth at the high shore. A previous study showed that only one out of three study sites indicated different growth rates between oysters with different immersion times (Paul-Pont et al. 2013), with highest growth at longer immersion time. Differences in growth of oysters and immersion times indicate the complexity of the growth mechanisms in oysters. Restricted growth in the short immersed oysters might be the result of too short immersion time to filter feed or a poor nutrient rich ecosystem. The lower mortality observed in "Resistant" French oysters, when held

at shorter immersion time, might be the result of the shorter exposure time of disease infected particles in the water (Peeler et al. 2012; Paul-Pont et al. 2013; Whittington et al. 2015), while the exposure time is still long enough to prevent starvation. Less exposure to diseases could support oysters to invest their energy in growth, resulting in healthy oysters, followed by reduced mortality. This restricted growth pattern for the "Naïve" Irish oysters may be the result of their higher vulnerability to diseases, and even with shorter exposure times, diseases might reduce their growth and induce mortalities.

A clear temporal pattern for prevalence of OsHV-1 µVar was observed with significantly higher prevalence in oysters from both families and age classes in the mid-summer sample date compared with the autumn samples at both shore heights. A significant temperature increase with temperatures reaching almost 20 °C were observed in the days before the first samples were taken in June 2017 (and might have activated the virus. The temperatures before the second sampling time (68 weeks post relaying) barely reached the threshold temperature of 16 °C (Clegg et al. 2014; Renault et al. 2014; Pernet et al. 2015), which might explain the differences in the presence of virus between the sample dates. In agreement, previous study suggested that infected oysters can clear the infection when exposed to colder temperatures (Petton et al. 2013).

In contrast to OsHV-1 µVar, no clear temporal pattern or spatial pattern was observed for *V. aestuarianus* prevalence, which highly suggests that disease development is temporal and that OsHV-1 µVar and *V. aestuarianus* might be active during different times of the year at this latitude. A previous Irish study found that mortality associated with *V. aestuarianus* mainly occurred in September and October (Ciar O'Toole at Annual NRL meeting on 28-30th of March 2017).

Our results suggest that oyster pathogens present at a culture site will not only affect age classes differently but also oyster families, with varying susceptibility to those oyster pathogens. The selectively bred oysters had higher prevalence of infection for both OsHV-1 µVar and *V. aestuarianus* for most samples, which were not associated with high mortality rates. However, the lower prevalence observed in the "Naïve" Irish oysters was most likely due to the very high mortality rates

that were observed in in this group and that uninfected survivors were being screened. Half grown oysters in both groups were more likely to demonstrate co-infections. Adult oysters have been reported to be more susceptible to *V. aestuarianus* (Travers et al. 2015; Green et al. 2016).

Our study indicated that overall, immersion time can be a factor in supporting reduced infection impact and mortalities associated with the presence of pathogens – however, this association can be complex and will vary with oyster stocks and associated environmental factors. Overall, in this study, selectively bred oysters performed better, in terms of growth and survival, but culture at high shore was preferable even in slightly worse growth conditions, due to the reduced mortalities. In conclusion we suggest to expose oyster seed to longer immersion time at low shore to reduce stressors induced by air exposure and support better survival. While we recommend to move up the half grown oysters to high shore to support better performance in growth. Overall this can provide an additional tool for farmers when trying to ameliorate the impacts of pathogens at culture sites particularly over months when most stressors are present.

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Chapter 6.

The effects of crude and refined algal derived antiviral bio therapeutics on ostreid herpesvirus development and Pacific oyster *Crassostrea gigas* performance

Abstract

Aquaculture species are often exposed to stressful holding and husbandry conditions, which can result in pathogen proliferation, disease development and subsequent mortalities in susceptible cultured species. Pro- and prebiotics in fish feed have been proven to support and enhance the immune system of marine species and their health inducing effects are of particular interest for the aquaculture industry. Sulphated polysaccharides (SP) are biocompounds derived from seaweed species, which have shown positive immune boosting effects in marine vertebrate and invertebrate species e.g. shrimps, but potential benefits areunknown for oysters. The Pacific oyster *Crassostrea gigas* is an important commercial aquaculture species worldwide. However, over the past few decades *C. gigas* stocks have been suffering summer mortalities related to a complex aetiology including disease causing pathogens such as ostreid herpes virus-1 (OsHV-1) and variant ostreid herpesvirus-1 microVar (OsHV-1 µVar) and bacteria *Vibrio spp*. This study investigated for the first time the effects of crude seaweed and extracted derivatives on the innate immunity of *C. gigas* and OsHV-1 µVar development. Crude seaweeds were used to determine how polycultures involving specific seaweeds might be used to reduce infection in co-located oyster stocks. Extracted derivatives were employed to see how a more concentrated compound could increase the efficacy of these substances, particularly at hatchery stages of production. Naïve and naturally exposed oysters (to both OsHV-1 µVar and *Vibrio* spp.) were held in the laboratory, with two seaweed species *(a) Fucus vesiculosus* and *(b) Mastocarpus stellatus*, found naturally attached to oyster PVC holding bags at oyster culture sites. In addition, in the laboratory, naturally exposed oysters were treated with one of two doses of an algal derived antiviral bio therapeutic for 8 days. Oysters in all trials were monitored for infection and immunological response. Results indicated that oysters exposed to either of the two seaweed species showed significantly lower prevalence of OsHV-1 µVar

compared to control oysters. Also, lysozyme activity was higher in both oysters held with seaweed species or treated with algal derived bio therapeutic products compared with the control oysters, indicating that exposure to these compounds was boosting natural defenses. However, over the limited study period no significant differences in oyster performance, like mortality and lysozyme activity were observed between the treatments and further research is recommended. These preliminary results indicate the importance of development of practical husbandry techniques of pacific oysters.

Key words

Aquaculture, *Crassostrea gigas*, ostreid herpesvirus-1 microVar, antiviral, sulphated polysaccharides

6.1. Introduction

Aquaculture can alter the ecology of fish and shellfish, thus promoting stress (Davis 2006), disease development (Murray & Peeler 2005) and even induce mortalities in cultured species (Sindermann 1984). Disease and pathogen outbreaks can occur due to (a) exposure of disease affected wild brood stock (Kurath & Winton 2011) and (b) intensive farming husbandry practices such as holding species in artificial environments (e.g. high stock densities, handling time, poor environmental circumstances etc.), which creates a stressful environment thus making species more sensitive to disease epidemics (Kent 2000; Walker & Winton 2010). Pathogens and disease outbreaks have been observed in many cultured marine species with many examples from such species as the flat oyster *Ostrea edulis*, penaeid shrimp *Penaeidae*, common carp *Cyprinus Carpio*, Nile tilapia *Oreochromis niloticus*, Golden Pomfret *Trachinotus blochii*, (Comps et al. 1980; Chou et. al. 1995; Hedrick et al. 2000; Prado et al. 2005; Dong et al. 2015; Chong et al. 2017) and the commercially important Pacific oyster, a species which is currently experiencing mortality events (Soletchnik et al. 1999; Cheney et al. 2000; Huvet et al. 2004; Bédier 2010; Mortensen et al. 2016; Prado-Alvarez et al. 2016).

Pacific oysters, *Crassostrea gigas*, have been suffering summer mortalities worldwide since the 1950's (Lynch et al. 2012). The cause of *C. gigas* summer

mortalities are suspected to be related to a complex aetiology, including interactions between the physical status of the host, environmental parameters such as water temperature (Malham et al. 2009; Chavez Villalba et al. 2010), dissolved oxygen levels (Cheney et al. 2000) (Chapter 5), eutrophication (Tamate et al. 1965; Malham et al. 2009), salinity (Luna-Gonzalez et al. 2008) and associated diseases and pathogens including ostreid herpesvirus -1 microVar (OsHV-1 µVar) (Chapter 4 and Chapter 5) and *Vibrio* spp. bacteria (Chapter 4 and Chapter 5) (Samain & McCombie 2008; Sauvage et al. 2009; European Food Safety Authority 2010; Lynch et al. 2012). The culture of marine species in open water makes the eradication of their pathogens and diseases almost impossible (Peeler & Otte 2014). New management tools like selective breeding programs of pathogen resistant species (Dégremont 2011), movement legislations (Renault 2011) and removal of host species (Ben-Horin et al. 2016) have been implemented but are labour intensive, time consuming or very expensive (Peeler & Otte 2014).

In recent years, the use of pro- and prebiotics in the diets of aquaculture species has increased (Cruz et al. 2013; Borch et al. 2015; Das et al. 2017). Pro and prebiotics have been shown to support the immune system and play a role in disease reduction and prevention. Pro- and prebiotics showed positive immune responses in marine vertebrates including fish species like; Atlantic cod *Gadus morhua*, Nile tilapia *Oreochromis niloticus* and Rainbow trout *Oncorhyncys mykiss*, when exposed to stressors (Gildberg et al. 1997; Kennedy et al. 1998; Lara-Flores et al. 2003; Panigrahi et al. 2010; Denev et al. 2010) and also in marine invertebrates including corals (Sweet et al. 2014), shrimp (Rico-Mora et al. 1998; Moriarty 1999; Meunpol et al. 2003) and scallops (Riquelme et al. 1997; Kesarcodi-Watson et al. 2012; Kesarcodi-Watson et al. 2016). In addition, probiotic treatment has been associated with improved performance in the larvae of *Crassostrea gigas* and the European flat oyster *Ostrea edulis* against different *Vibrio* spp. bacteria (Kesarcodi-Watson et al. 2012).

Molluscs, like Pacific oysters, relay on their innate immune system as they are lacking specific immune response and immunological memory. The environment can directly and/or indirectly affect the innate immune system of marine invertebrates (Galloway & Depledge 2001; Mydlarz et al. 2006). Their innate

immune responses and mechanisms involve immune cells, genes and proteins (Allam & Roftos 2015) and is responsible for providing resistance to microbial pathogens. It is pivotal in preventing (or not) disease outbreaks (Medzhitov & Janeway 1997). Vaccination against infectious pathogens and diseases in these species therefore are not possible due to dependence on the innate defences (Gestal et al. 2008).

Sulphated polysaccharides (SP) are biocompounds found in seaweed species (Percival 1979), which have been shown to boost the health of invertebrates by their wide variety of immune stimulating, anticancer, antiviral and antibacterial activities (Deig et al. 1974; Wright et al. 1981; de Almeida et al. 2011; Rudtanatip et al. 2018). Algal species have their own specific range of different algae-derived polysaccharides, which differ between brown and red algae seaweed species. Brown algae seaweeds contain a sulphated polysaccharide called fucoidan (Kawishima et al. 2011; Isnansetyo et al. 2016), (1,3)-β-d-glucan (Brown & Gordon 2005) and laminarin (Holdt & Kraan 2011; Smith et al. 2011). Fucoidan has been successfully used in Asian aquaculture involving the Whiteleg Shrimp *Litopenaeus vannamei* against infection with white spot syndrome virus (Wongprasert et al. 2014; Sinurat et al. 2016) and in fish Nile Tilapia *Oreochromis niloticus* infected with *Streptococcus sp.* infection (Isnansetyo et al. 2016). A previous study by Cipriano-Maack et al. (2016) investigated the immunomodulatory effects of a diet consisting of the brown seaweed *Laminaria digitata* on the edible sea urchin, *Paracentrotus lividus*. The findings of that study concluded that a *L. digitata* diet may impact immune parameters such as nitric oxide boosting immune response (Cipriano-Maack et al. 2016). Red algal seaweeds contain multiple sulphated polysaccharides, for instance galactan sulphate (Craigie 1990) and carrageenan (McCandless & Craigie 1979), and are well known to be a successful antiviral drug in the treatment of herpes virus and Influenza A in humans (Ehresmann et al. 1977; Richards et al. 1978). Moreover, when Asian tiger shrimps *Penaeus monodon* were treated with a diet extracted from red seaweed species *Asparagopsis* spp., it showed a significant effect on the control of *Vibrio* infection (Manilal et al. 2012).

Along the Irish coastline, common seaweed species include the brown seaweed species *Fucus vesiculosus* and the red seaweed species *Mastocarpus stellatus* (Hardy & Guiry 2003). Previous studies indicated that the mineral content of both species, including sulphate concentrations varied between 2.4−11.5 g/100 g dry weight (Rupérez et al. 2002) and 3.75g ±0.04 /100g dry weight (Rupérez 2002) for *Fucus vesiculosus* and 0.6–3.9 g/100 g dry weight for *Mastocarpus stellatus* (Gómez-Ordóñez et al. 2014).

Nowadays there is a new market of commercial products containing immunomodulating marine sulphated polysaccharides (MSPs), vitamins and amino acids (concentration unknown) to support immune system performance in farmed and cultured animals. Those products are recommended when species face challenging circumstances like internal and external stress and lower immune defenses and to support the natural defences in vertebrates (IRTA 2015). It is suggested that MSPs might support either innate or adaptive immune systems (Berri et al. 2016). However, there is not much known about their impacts on bivalves, and specifically *C. gigas*.

Lysozyme is an enzyme linked with natural defence and is suggested to be an indicator of physiological condition and vitality (Chu & La Peyre 1989). It is found in different organisms including animals, plants and bacteriophages (Jolles & Jolles 1984) and has also been detected in oysters (Mcdade & Tripp 1967; Cronin et al. 2001; Prado-Alvarez et al. 2015). Lysozymes can support the immune system by lysing cell walls of invading particles, which lead to osmotic and mechanical stress in the cell and often death (Ganz 2003).

The initial objective of this study was to investigate the effects of two seaweed species *(a) Fucus vesiculosus* and *(b) Mastocarpus stellatus*, commonly found in abundance at Irish *C. gigas* culture sites attached to oyster holding bags on oyster mortality, ostreid herpes virus-1 microVar and *Vibrio aestuarianus* development and immune response in *C. gigas.* Effects were tested on naïve (Trial 1) and naturally pathogen exposed *C. gigas* collected from an ostreid herpes virus-1 microVar and *Vibrio aestuarianus* endemic site (Trial 2). The second objective was to investigate the health effects of an algal derived commercial product on

naturally exposed Pacific oysters to the pathogen collected at the same endemic site as the first study (Trial 3). For both studies, oysters were held under an increasing temperature regime which is often experienced over the summer months, to test the effects when held under stressful circumstances. Overall, the study aimed to determine if these compounds could be used to reduce impact of these two pathogens under culture conditions.

6.2. Material and methods

(1) Study 1. Effects of the potential physiological and immunomodulatory impact of two seaweed species on oysters impacted by OsHV-µvar and Vibrio aestuarianus.

In Study 1, two trials were carried out: a) to assess the impact of exposure to seaweed on the immune system of oysters that had not previously been exposed to herpes virus or vibrios (Trial 1); and b) a trial to assess the impact of the seaweed on oysters from an area where both pathogens were endemic (Trial 2).

Oysters

In Trial 1, naïve oysters were used from a hatchery in New Quay, Galway Bay (at New Quay, Galway Bay (53.1545 °N, 9.0828 °W), Ireland. Oysters had an average weight of 14.1 gram ± 3.57 (SD) with a weight range of 1.43 - 24.66 gram and an average length of 52.7 mm ± 6.7 (SD) with a length range of 36.6 - 73.7 mm.

In Trial 2, oysters were collected from a *C. gigas* culture site in Dungarvan, Co. Waterford (52.0936 °N -7.6204°W) in September 2016. This culture site has a history of OsHV-1 µvar and *Vibrio aestuarianus* and therefore oysters are naturally exposed to oyster diseases. Oysters had an average weight of 2.96 gram ± 1.19 (SD) with a weight range of 0.35 - 7.36 gram and an average length of 28.53 mm ± 4.86 (SD) with a length range of 17.2 - 44.1 mm.

Seaweed species

Seaweed species that were both common and abundantly attached to oyster PVC holding bags at Irish Pacific culture sites were used. The two seaweed species at

the culture site were removed from the bags and consisted of a brown seaweed *Fucus vesiculosus,* and a red seaweed species *Mastocarpus stellatus* (Figure 1).



Figure 1. Seaweed species Fucus vesiculosus (left) and seaweed species Mastocarpus stellatus (right)

Previous studies indicated a sulphate concentration of 2.4−11.5 g/100 g dry weight (Rupérez et al. 2002) for *Fucus vesiculosus* and 0.6–3.9 g/100 g for *Mastocarpus stellatus* (Gómez-Ordóñez et al. 2014). In this study sulphate concentrate was not analysed, but the amount of seaweed used was compatable to the seaweed density attached to the oyster trestle bags in the field. After collection of the seaweed species in the field from an OsHV-1 µVar and *V. aestuarianus* endemic culture site, the seaweed was washed with tapwater to remove all attached organisms and any potentially attached pathogens. A total of 100 g seaweed (water squeezed out and blotted dry) was used per experimental tank (8 l).

Experimental set up

The experimental set up for both Trial 1 and Trial 2 consisted of nine stand-alone tanks consisting of 10 L plastic aquaria with 8 L of UV treated seawater aerated with an air stone at a salinity of 34 with temperature of 14 °C – 19°C (gradually over a time span of 24 hours) and exposed to 12 hours of darkness (8pm – 8 am) and 12 hours of light (8 am – 8pm). The nine tanks were divided into replicates of 3 tanks per treatment: one treatment of three control tanks (no seaweed), one treatment of three tanks with seaweed *F. vesiculosus* and one treatment with three

tanks with seaweed *M. stellatus.* No water exchange took place. Thirty oysters were used per tank, giving 90 control oysters and 90 oysters per treatment (seaweed *F. vesiculosus* and *M. stellatus*) in total. Both Trial 1(a) and 1(b) ran for 26 days (Day 1 – Day 26) and during the first 19 days the oysters were exposed to 14 °C, after Day 19 the temperature went up to 19 °C for 7 days (Day 26) to apply a thermal shock to the oysters and encourage viral replication in the system if present.

Prior to the trial commencing, an initial sample of 30 oysters was screened for pathogens. The oysters were checked for mortality several times daily and any moribund or dead individuals were removed immediately and processed. On days 2, 5, 7, 12, 19 and 22 three oysters per tank were randomly selected and processed, resulting in a total of 9 oysters per treatment at fixed time points during the trial. All remaining oysters dead or alive were processed on the final day of the trial (Day 26). No water changes were conducted for the duration of the trial and oysters were fed 2 ml of Shellfish Diet 1800 (Reed Mariculture, A mixed diet of *Isochrysis*, *Pavlova Thalassiosira*, *Tetraselmis*, size: ~5-12 microns, dry weight 8%, more information: www.shellfish-diet.com) was provided at Day 7, 14 and 21.

(2) Study 2. Effects of a commercial algal derived product on oysters naturally exposed to OsHV-1 microVar and Vibrio aestuarianus

In Study 2, the health effects of a commercial animal health product Searup from Olmix Group (www1) on oysters from an area where both pathogens were endemic were studied (Trial 3).

Oysters

C. gigas were collected from Dungarvan, Co. Waterford (52.0936 °N -7.6204°W) in November 2016. Oysters had an average weight of 4.06 gram ± 2.18 (SD) with a weight range of 0.19 – 11.24 gram and an average length of 30.85 mm ± 6.75 (SD) with a length range of 13.7 – 52.0 mm.

Experimental set up

A similar set up of tanks was used as in Study 1 (Trial 1 and 2) and two treatments of Searup were used. The two treatments (dose, duration and temperature regime) were developed in consultation with a veterinarian representative of the company. In Searup Treatment 1, the experimental oysters were exposed to the product i.e. 2 ml/L seawater per day for the first 3 days, which gave a total of 16 ml (2 ml x 8 liter = 16 ml per tank) per day for the first 3 days of the trial resulting in an overall total of 48 ml per tank for the trial duration. In Treatment 2, experimental oysters got a single double dose of the product (4 ml/L) on Day 1 resulting in the oysters exposed to a total of 32 ml per tank for the trial duration. The trial ran for 8 days with the oysters being held at 16 °C for the first five days of the trial and subsequently at 19 °C from Day 6 until Day 8. The oysters were checked several times daily for mortality and any moribund or dead individuals were removed and processed. On each day, Day 1-7, three oysters per tank were randomly selected, processed and screened. All remaining oysters were processed on the last day, Day 8 and screened.

This trial was repeated again in 2017, exactly as the previous trial with this product. Oysters were collected from Dungarvan, Co. Waterford (52.0936 °N - 7.6204°W) in September 2017. Oysters had an average weight of 3.68 gram ± 0.83 (SD) with a weight range 1.90 – 6.88 gram and an average length of 34.2 mm ± 2.93 (SD) with a length range of 26.8 - 42.0 mm.

(3) Processing of individuals

Morphometric characteristics weight (g) and length (mm), of each oyster, were recorded using a balance scales and vernier calipers. For detection of oyster pathogens, oyster gill tissue (approximately 5mm^2) was taken for DNA extraction (chelex) to screen for ostreid herpesvirus-1 microVar and *V. aestuarianus*. For indication of performance of the immune system, haemolymph was collected to investigate lysozyme levels and activity. Haemolymph (100µl) was removed from the pericardial cavity with a needle and syringe. Blood for the lysozyme assay was placed in 100µl of EDTA to prevent coagulation. Haemolymph was centrifuged at 3000g for 10 min. The serum, cell free component, was removed and placed in 1·5 ml eppendorf tubes and stored at – 20 °C until further diagnostics took place.

(4) Molecular diagnostic screening

DNA extraction

DNA extraction was performed using the Chelex-100 methodology (Walsh et al. 1991). Gill tissue samples (approx. 5mm^2) were placed in a 10% chelex solution (100 microliters volume) (Sigma Aldrich), placed in a Hybaid thermal cycler for 1 hour and 10 minutes and heated at 99 °C to facilitate cell lysis (Walsh et al. 1991).

Polymerase chain reaction (PCR) for OsHV-1 µVar

Standard PCR (PCR) was initially carried out to detect OsHV-1 µVar in the gill of the *C. gigas* by following protocol of Lynch et al. 2013 by using OHVA/OHVB primers (Lynch et al. 2013). All PCRs used a total of 2 µL genomic DNA template per individual. Expected size of amplified PCR products for OsHV-1 µVar was 385 bp and PCR was carried out in 25 µL containing 12·9 µL dd$H_2$0, 5 µL, 5× buffer, 5 µL dNTPs (0·2 mM), 0·5 µL $MgCl_2$ (25 mM stock), 0·25 µL of each primer (100 pmol mL^{-1} stock) and 0·1 µL Taq DNA polymerase. Positive controls (duplicate) consisting of OsHV-1 µVar infected oyster tissue and negative controls (duplicate) of double distilled water (ddH_2O) were used for each PCR. Thermo cycling conditions were performed by initial denaturation of 1 min of 95 °C, following by 35 cycles including a denaturation step of 20 seconds at 94 °C, an annealing step of 30 seconds at 56 °C and an elongation step at 72 °C and finishing with a final elongation step of 7 minutes at 72 °C by using a thermo Hybaid PCR express thermal cycler (Lynch et al. 2013). Presence of amplified PCR products was confirmed by electrophoresis using a 2% agarose gel stained with ethidium bromide (10mg/l stock) and was run with an electrical charge of 110V for 45-60 minutes.

Quantitative polymerase chain reaction (qPCR) for OsHV-1 µVar

Quantitative PCR (qPCR) was carried out to determine the viral load of samples deemed positive for OsHV-1 µVar by PCR and followed out by the protocol "OsHV-1 detection and quantification by real Time Polymerase Chain Reaction using OsHV-1 DNA polymerase sequence" (www[7]) using primers HVDP-F and HVDP-R (Webb et al. 2007). All qPCRs used a total of 5 µL genomic DNA template per individual (duplicate). The qPCR mix was carried out in 25 µL

containing 12.5 µl 2 x Brilliant Sybr Green ® Q PCR Master Mix, 2.5 µl HVDP-F (5µM) and 2.5 µl HVDP-R (µM) primers and 2.5 µl ddH_2O. Standards were used to detect the exact amount of viral copies in tested samples. Standard curves were prepared by diluting a viral DNA suspension of 10^8 viral copies of OsHV-1 (supplied by IFREMER). qPCR plates included 5 dilutions of 10^5, 10^4, 10^3, 10^2 and 10^1 viral copies. Thermo cycling conditions were performed by initial denaturation of 2 min of 50 °C and 10 min at 95 °C, following by 40 cycles of 15 seconds at 95 °C and 1 min at 60 °C and a melt curve of 95 °C for 15 seconds, 60 °C for 1 minute, 95 °C for 30 seconds and 60 °C for 15 seconds by using a thermo Hybaid PCR express thermal cycler.

Quantitative polymerase chain reaction (qPCR) for Vibrio aestuarianus

Quantitative PCR (qPCR), following the protocol of McCleary and Henshilwood (2015)**,** was carried out to determine *V. aestuarianus* load. qPCR was performed in duplicate using 5 µl DNA. The master mix consisted of 12.5 µl 2xTaqmanUniversal master mix, 0.75 µl Forward *dnaJ* f420 primer (10µM), 0.75 µl *dnaJ* r456 Reverse primer (10µM), 0.38 µl *dnaJ* p441Probe (10µM) and 5.62 µl dH_2O. Thermo cycling conditions were performed by an initial stage of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s by using a thermo Hybaid PCR express thermal cycler (McCleary & Henshilwood 2015). Negative template control consisting of ddH_2O and positive controls (duplicate), oyster tissue infected with *V. aestuarianus* (supplied by Marine Institute Ireland) were included on each plate.

Immunoassay: lysozyme analysis

Analysis of lysozyme levels and activity were performed following the protocol of Cronin et al. (2001) according to Caraballal et al. (1997). The haemolymph serum was defrosted and 30µl of serum was placed in triplicate into a 96 well round flat-bottomed plate. 170 µl of the *Micrococcus lysodeikticus* suspension (0.2 $mg.ml^{-1}$ sodium phosphate buffer pH 6.4, store at –20^oC) was added to the serum and the decrease in absorbance was recorded at 450 nm every minute for the following 4 minutes using a Spectra III SLT plate reader (Cronin et al. 2001; Caraballal et al. 1997).

In our study, Optical Density (OD) absorption levels were used to represent lysozyme as lysozyme (the difference in absorption at T^0 and T^4 (Figure 2). OD absorption can vary between 0 and 1. Absorption levels were measured at T(0) min and T(4) min. A low absorbance value denotes a high lysozyme amount while a high absorbance value denotes a low lysozyme amount.

Lysozyme activity

OD T(0) – T(4): Positive difference when absorbance is reduced over time which indicates less bacteria left and therefore lysozyme activity.

OD T(0) – T(4): No difference when absorbance did not show difference over time which indicates no lysozyme activity.

The larger the positive difference in absorbance, T(0) – T(4), the more bacteria is reduced, the higher the lysozyme activity.

Figure 2. Analyses of lysozyme activity

(5) Statistical analyses

Oyster mortality and prevalence of infection were analysed by Chi-square test. Normality was tested using the Shapiro-Wilks Normality test. Differences in lysozyme amount and activity were tested by Student's t-test. For both tests, significant differences at $P \leq 0.05$ were considered.

6.3. Results

Study 1. Trial 1. Effects of the potential physiological and immunomodulatory impact of two seaweed species on naïve oysters

Mortality

In Trial 1, mortality was only observed on Day 5 for a single oyster exposed to *F. vesiculosus* (1.1%, n=1/90).

Pathogen (OsHV-1 microVar and Vibrio aestuarianus) prevalence

The initial sample of the naïve *C. gigas* (n=30) was negative for OsHV-1 µVar and *Vibrio aestuarianus* and neither OsHV-1 µVar nor *V. aestuarianus* was detected in either the control or the seaweed exposed oysters.

Lysozyme activity

Naïve oysters showed lysozyme activity at the initial sample. Average OD absorption was 0.0025 for *F. vesiculosus* treatment, 0.0009 for *M. stellatus* treatment and 0.0015 for control oysters. From day 5 oysters treated with *M. stellatus* and control oysters did not indicate any lysozyme activity anymore, while oysters treated with *F. vescilosus* indicated lysozyme activity at day 5, day 19 and day 22 (Figure 3).

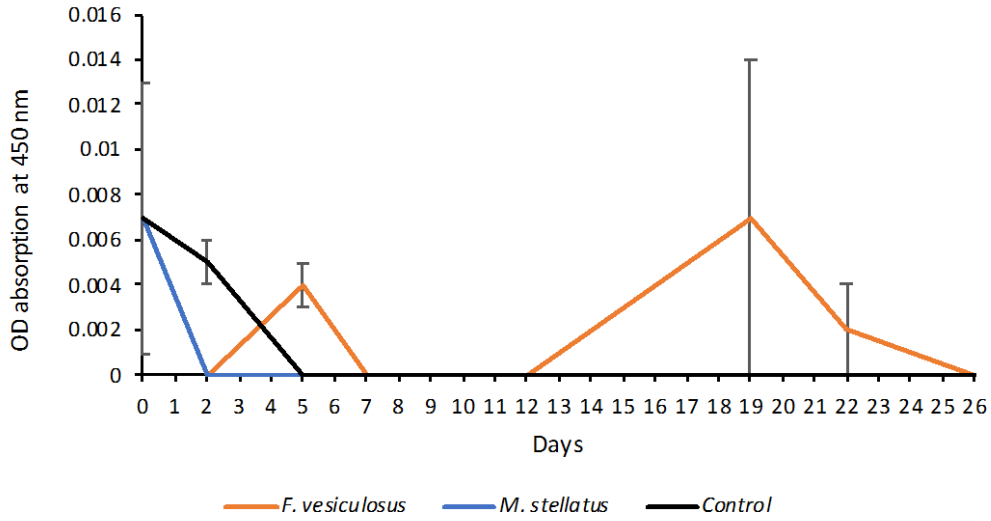


Figure 3. Average (+SE) OD absorbance indicating lyzozyme activity at day 0 (initial sample), 2, 5, 7, 12, 19, 22 and 26 in different treatments in study 1 trial 1.

Study 1. Trial 2. Effects of the potential physiological and immunomodulatory impact of two seaweed species on oysters impacted by OsHV-µvar and Vibrio

aestuarianus of oysters naturally exposed to OsHV-1 microVar and Vibrio aestuarianus

Mortality

Mortality in the virus-exposed oysters for all treatments, seaweed exposed oysters and control oysters, during the trial remained low at < 5% with 1.1% (n=1/90) in control oysters, 1.1% (n = 1/90) in oysters exposed to *F. vesiculosus* and 4.4% (n = 4/90) in oysters exposed *M. stellatus.*

Pathogen (OsHV-1 microVar and Vibrio aestuarianus) prevalence

The initial oyster sample from the *C. gigas* culture site had a prevalence of 53.33% for OsHV-1 µVar and 0% for *Vibrio aestuarianus.* No *Vibrio aestuarianus* was observed during the whole trial. The seaweed treatments had a OsHV-1 µVar prevalence of 22.5% over the whole trial, with a OsHV-1 µVar prevalence of 22.0% for oysters exposed to *F. vesiculosus* and 23.1% for oysters exposed to *M. stellatus.* Control oyster indicated a significant higher OsHV-1 µVar prevalence of 39.8% compared with both seaweed treatments (X^2 = 9.1, df = 2, P = 0.01). Overall pattern indicated a high prevalence (> 50%) for oysters exposed to either of the seaweed species at the beginning of the trial, which reduced over time. Oysters from the control treatment had consistent infection levels throughout the study (Figure 5).

qPCR was performed on a subsample (n=82 out of 92 positives screened by PCR) of oysters, which indicated prevalence of ostreid herpesvirus-1 micorVar in the standard PCR. Average viral copies of $1.7.10^2$ (n =16) was found for the initial sample. Viral loads of experimental oysters (n = 66) over time for all treatments of oysters are illustrated in Figure 4 and Table 1.

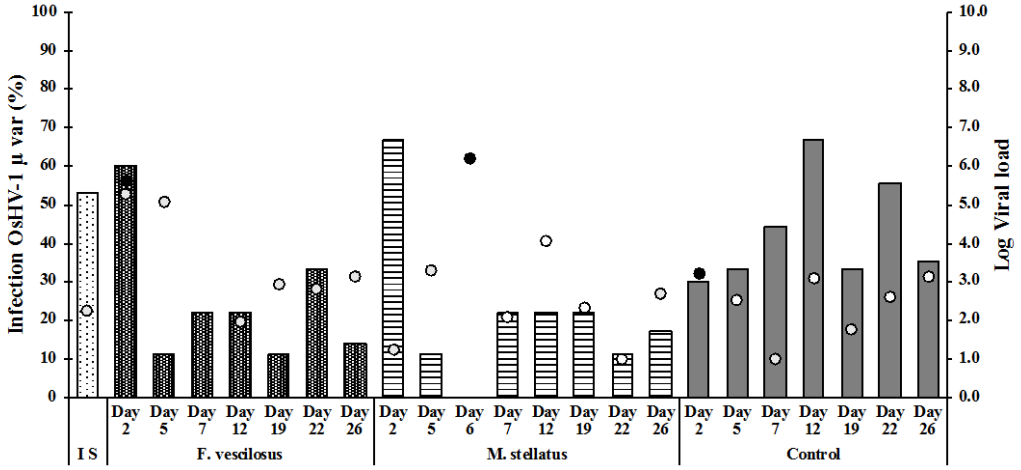


Figure 4. Prevalence of ostreid herpes virus-1 microVar and their viral load (IS: Initial sample, white round marker: living oysters, black round marker: dead oysters) in oysters exposed to seaweed species in study 1 trial 2 (n=9 per day per treatment)

Table 1. Average viral copies for different treatments in study 1 trial 2. (n= number of oysters screened)

Day	*F. vesiculosus* Oyster alive	*F. vesiculosus* Oyster dead	*M. stellatus* Oyster alive	*M. stellatus* Oyster dead	Control Oyster alive	Control Oyster dead
2	$1.8.10^5$ (n=1)	$4.2.10^5$ (n=1)	$1.7.10^1$ (n=4)		$1.6.10^3$ (n=1)	$5.0.10^3$ (n=3)
5	$1.2.10^5$ (n=1)		$1.9.10^4$ (n=1)		$3.3.10^2$ (n=3)	
6				$1.6.10^6$ (n=2)		
7			$1.2.10^2$ (n=1)		$1.0.10^1$ (n=2)	
12	$9.1.10^1$ (n=2)		$1.2.10^4$ (n=2)		$1.2.10^3$ (n=6)	
19	$8.6.10^2$ (n=1)		$2.1.10^2$ (n=1)		$6.0.10^1$ (n=3)	
22	$6.3.10^2$ (n=3)		$<1.0.10^1$ (n=1)		$3.9.10^2$ (n=5)	
26	$1.3.10^3$ (n=5)		$5.1.10^2$ (n=6)		$1.4.10^4$ (n=11)	

Lysozyme activity

No lysozyme activity was detected in initial samples of non-infected exposed oysters (Figure 5) and infected exposed oysters (Figure 6). Non-infected oysters indicated lysozyme activity at day 7, day 22 and day 26 when treated with *F. vescilosus*. Infected oysters had lysozyme activity at day 5 and day 22 when treated with M. *stellatus* and at day 19 and 22 when treated with *F. vescilo*sus. No lysozyme activity was detected in control oysters indicated for both non-infected and infected oysters.

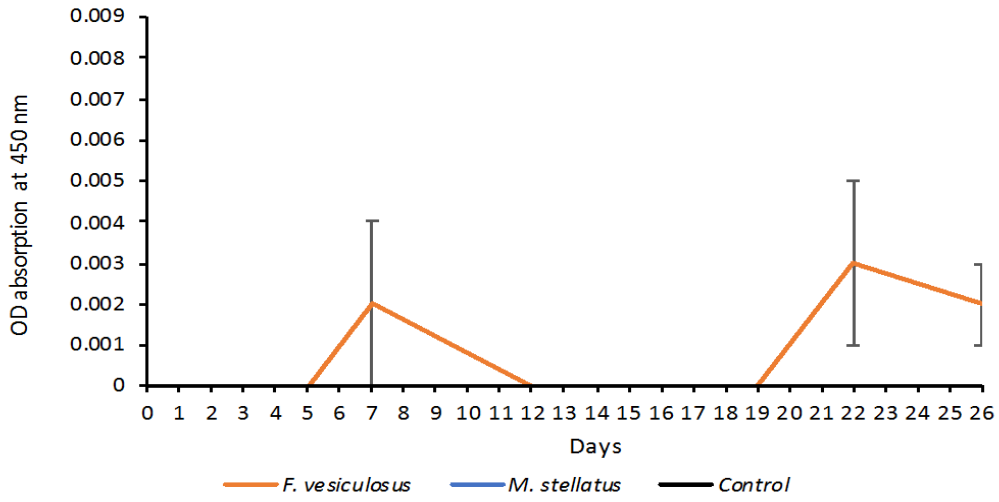


Figure 5. Average (+SE) OD absorbance indicating lyzozyme activity for non-infected oysters exposed to different treatments activity at day 0 (initial sample), 2, 5, 7, 12, 19, 22 and 26 in study 1 trial 2.

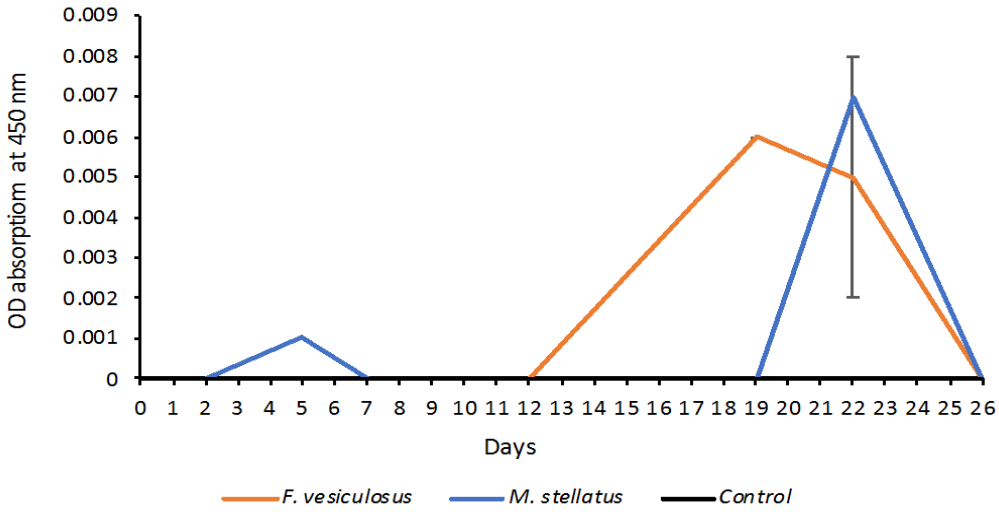


Figure 6. Average (+SE) OD absorbance indicating lyzozyme activity for infected oysters exposed to different treatments at day 0 (initial sample), 2, 5, 7, 12, 19, 22 and 26 in study 1 trial 2.

(2) Study 2. Effects of a commercial algal derived product on oysters naturally exposed to OsHV-1 microVar and Vibrio aestuarianus

Mortality

Oyster mortality remained low (< 5%) in Treatment 1 of the commercial product (4.4%, n = 4/90) and the control treatment (3.3%, n = 3/90), while oysters in Treatment 2 of the commercial product had a higher mortality rate (11.11%, n = 10/90). When this trial was repeated, no mortality was observed within any treatment over the whole experiment.

Pathogen (OsHV-1 microVar and Vibrio aestuarianus) prevalence

OsHV-1 µVar and *V. aestuarianus* was not detected in the initial sample and no pathogens were observed throughout the duration of the trial even with the temperature increase. When this trial was repeated, again no pathogens were observed in the initial sample and experimental oysters.

Lysozyme activity

Lysozyme activity was observed at both treatments and control oysters. Average OD absorption was 0.00334 for Treatment 1 of commercial product, 0.00333 for Treatment 2 of commercial product and 0.00067 for control oysters. Control oysters only indicated activity at the first days, while product treatments showed more activity during the trial (Figure 7).

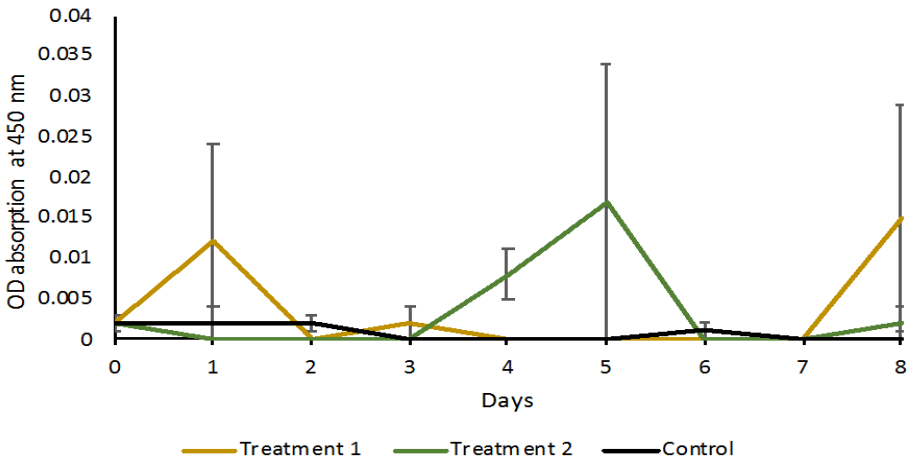


Figure 7. Average (+SE) OD absorbance indicating lyzozyme activity for individuals in different treatments at day 0 (initial sample), 1, 2, 3, 4, 5, 6, 7 and 8 in Study 2.

6.4. Discussion

Fouling of oyster PVC bags by multiple seaweed species is a common occurrence at culture sites (Pers. Obs.), however, the exact effects of this fouling on oyster health has been uncertain and is generally thought to have a negative impact by the industry, possibly inhibiting optimal feeding and respiration by the oysters. The findings of this study suggest a positive effect of exposure of oysters to seaweed species with an anti microbial effect on Pacific oyster pathogens. To the best of our knowledge, no study has been carried out to determine the effects of naturally degrading seaweed exposure on a marine species. Diets of seaweed extracts (Wongprasert et al. 2014; Sinurat et al. 2016, Isnansetyo et al. 2016; Manilal et al. 2012) and seaweed species (Cipriano-Maack et al. 2016) have been successfully used as an immune stimulant for aquaculture species like, penaeid shrimp *Penaeidae* (Wongprasert et al. 2014; Sinurat et al. 2016), tilapia *Oreochromis niloticus* (Isnansetyo et al. 2016) and edible sea urchin, *Paracentrotus lividus* (Cipriano-Maack et al. 2016).

Disease development

In the whole study, no *V. aestuarianus* was detected and therefore the potential impact on this pathogen could not be detected.

In Study 1 Trial 1 (naïve oysters with seaweed treatment), mortalities remained low and no significant mortalities were observed between treatments and control oysters for naïve oysters. No herpes virus was detected, suggesting that besides mortality no pathogens were introduced by holding naïve oysters with seaweed species. In addition, no significant differences were detected for lysozyme amount between naïve oysters exposed to either of the seaweed species and oysters for control treatment. Lysozyme activity was mostly observed in oysters treated with *F. vescilosus* suggesting there is a possible immune boost possible.

In Study 1 Trial 2 (naturally pathogen exposed oysters with seaweed treatment), mortality was observed and highest viral load was associated with dead individuals. However, no significant mortalities were observed between treatments and control oysters when oysters were naturally pathogen exposed. Oysters treated with seaweed species showed a higher prevalence of the virus at

the start of the trial compared to oysters from the control treatment. A lower prevalence of infection of herpes virus in seaweed treated oysters was observed from day 5 onwards. Overall when temperatures increased, oysters exposed to either seaweed species indicated a suppression of the virus, and over the whole trial, prevalence of infection was significantly lower in oysters exposed to seaweed species compared with control oysters. A recent study indictaed higher lysozyme levels in mussels after exposure to sulfated polysaccharides extracts of European red seaweed Irish moss *Chondrus crispus* (Rudtanaip et al. 2018). This supports the increased performance of oysters when exposed to natural degraded of seaweed species in our study.

In Study 2 (naturally pathogen exposed oysters with treatment of commercial product)**,** unfortunately, due to lack of herpes prevalence, this study can give no conclusions about the effects of the commercial algal derived product trial. As algal derived products have been successful stories in increasing non-specific immune response in other marine species (Wongprasert et al. 2014; Isnansetyo et al. 2016; Sinurat et al. 2016), more research on immune boost characteristics by commercial products on virus exposed oysters is highly recommended.

Lysozyme activities

Overall our lysozyme activities from serum samples have been low. Low levels of activity are in agreement with a previous study by Cronin et al. 2001, where *C. gigas* showed lower lysozyme activity compared with European Flat oyster *Ostrea edulis*. Moreover previous studies showed higher detection of lysozyme in cell than in serum (Hawkins et al. 1993; Carballal et al. 1997). Lysozyme activities were higher in oysters exposed to commercial product (Study 2) compared with oysters exposed to seaweed species (Trial 1 and Trial 2 of Study 1).

Lysozyme activities (Trial 1 and Trial 2 of Study 1) were mainly observed in experimentally treated oysters and not in control oysters. Between the seaweed species, *F. vescilosus* induced higher lysozyme activity. An explanation could be the related sulphate concentrations of the seaweed species, previous studies indicated a higher sulphate concentration of 2.4−11.5 g/100 g dry weight (Rupérez

et al. 2002) for *Fucus vesiculosus* compared to *Mastocarpus stellatus* 0.6–3.9 g/100 g dry weight (Gómez-Ordóñez et al. 2014).

Of note, in Study 2 lysozyme levels were significantly higher than lysozyme levels in Study 1 (both for Trial 1 and Trial 2). For the first three days of Study 2, oysters treated with a single dose of commercial product had the highest lysozyme amount and therefore it suggests that commercial product might stimulate the lysozyme production in the best way.

These results suggested that both seaweed species and the commercial product might act as immunostimulants by increasing lysozyme activity. In agreement, a previous study indicated that immune stimulants could affect lysozyme activities in *Ostrea edulis* (Prado-Alvarez et al. 2015). An increase in lysozyme activity in oyster *Ostrea edulis* after exposure of immune stimulants Lipopolysaccharide (LPS), zymosan and curdlan for both naïve and *Bonamia ostreae* infected *O. edulis*. This increase in lysozyme was however only significant up to 24 hours post immune stimulation. At the same time prevalence level of infection of the pathogen *B. ostreae* remained lower when exposed by water borne administration to those immune stimulants (Prado-Alvarez et al. 2015). The higher lysozyme activity in oysters exposed to commercial product, suggesting quicker access to compounds from extract rather than whole plant.

No correlation between infection of herpes virus and lysozyme activity was detected. Another study detected a suppression of lysozyme in Eastern oyster *Crassostrea virginica* when individuals were infected with *Bucephalus* and *Minchinia* (Feng & Canzonier 1970) and *Haplosporidium nelsoni* (La Peyre 1993). However, another studies did not find a correlation between lysozyme and disease in the European Flat oyster *Ostrea edulis* infected with parasite *Bonamia ostreae* (Cronin et al. 2001: Prado-Alvarez et al. 2015). Therefore, the actual role of lysozyme in host defence of *C. gigas* still remains unclear and needs to be considered with other immune parameters.

Experimental design

Our experiment took place under experimental conditions therefore, seaweed was exposed to artificial light source. Natural light plays a crucial role in physiology and ecology of plants, including growth patterns and resource allocation. The precise effect on their physiology and ecology caused by artificial light is complex and acquires a wide spectrum of information like light intensity, distribution and timing (Bennie et al. 2016). We tried to simulate a natural environment to expose the oysters to 12 hours light (8am-8pm) and 12 hours dark (8pm-8am) management. However, seaweed slowly started to degrade over the study, which could have increased the natural leaking or production of bio compounds. Further investigation would be necessary to ascertain the possible effect of seaweed species on oyster pathogen development and performance under natural circumstances.

Conclusions

In conclusion, our results suggest a possible positive effect of treatment with seaweed species on disease prevalence of naturally pathogen exposed Pacific oysters under laboratory circumstances. Moreover, both seaweed species and algal derived commercial products impacted on lysozyme activities positively. It would be of interest to carry out further studies to test new seaweed species, shorter/longer exposure times, different seaweed concentrations and natural conditions and to test commercial algae derived products on virus infected oysters. From an oyster culture perspective, these results support the development of a biotherapeutic treatment to boost the health of imported spat and seed after stressful periods caused by transport or handling procedures to give them a head start at their new culture site. Spat and seed could be held at covered ponds onshore, if present,with seaweed species or it may also be a treatment option in hatcheries to boost broodstock and larvae and reduce/eliminate pathogens.

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Chapter 7.

Discussion and conclusions

A summation of this PhDs contribution to a better understanding of oyster pathogens:*Crassostrea gigas:*environment relationship and dynamics

Although substantial research efforts have gone into better understanding of Pacific oyster mortalities worldwide, a lot of questions remain unanswered regarding the mechanisms of how pathogens associated with such events are becoming established at these sites and potentially outside their host species. The present study was undertaken to fill those significant knowledge gaps and to gain a better understanding of the abiotic and biotic drivers and inhibitors of OsHV-1 μvar and *Vibrio aestuarianus*.

From the findings of this study we now know that OsHV-1 μvar and *Vibrio aestuarianus* can be present in other cohabiting species that play diverse ecological roles, such as crabs, cockles and mussels at oyster culture sites. Not only are both pathogen species present in those species at culture sites, but they can also be found in mussels <30 km from "hot spots" of infection i.e. oyster farms. No research was performed further than 30 km from "hot spots" of infection, therefore it is unclear if those pathogens might spread in the environment up to >30 km outside these "hot spots".

In addition, this study demonstrated that crabs and cockles can act as carriers and/or reservoirs of infection transmitting pathogens to naïve oysters within a short period of time. This indicates that the virus and bacteria are exhibiting some degree of plasticity in how they can spread within the system and suggest the potential of other marine invertebrates functioning as carriers and reservoirs of OsHV-1 μvar and *Vibrio aestuarianus.* To gain better understanding of these transmission dynamics, I would recommend additional laboratory trials with more stressful conditions like increased temperatures, higher initial prevalence of pathogens and exposing naïve crabs/cockles to infected oysters.

The mechanisms of such oyster pathogen dispersal modes from culture sites require further analyses, however, preliminary results would indicate that both pathogens expand their range via water currents. Indeed, hydrodynamic site specific models previously have been successfully used to derive estimates of potential risk from hydrodynamic transport of pathogens released by other aquaculture practices in the marine environment. For future research I would recommend to focus on pathogen prevalence in water samples (sediment and zooplankton) and viability of pathogens outside their host. This information in combination with specific site hydrodynamic models could be of significant importance to forecast possible disease outbreaks,

Of interest, the prevalence of OsHV-1 μvar was often greater in crabs and cockles compared to the Pacific oysters, while *V. aestuarianus* although present in those species was less abundant. This would highlight that the two oyster pathogens may employ a different epidemiology and that the pathogens are jumping host from a less susceptible (disease resistant bred) oyster host species to other species within its environment, that may act as carriers or reservoirs of infection. It was not determined in this study if both pathogens cause mortalities in crabs, cockles and mussels and what those repercussions would have both from an ecological and commercial point of view, however, future work in this new research area could carry on from the findings of this study. This could easily be investigated under laboratory conditions by inoculating of different viral/bacterial load into naïve species and record their mortality rate over time.

So we have learnt a lot about the mechanisms that these pathogens use: oyster pathogens showed plasticity, viability and persistence in the marine environment. It raises some interesting questions about their ability to replicate in various hosts and their plasticity and how hosts need to evolve to respond to these ever-changing threats. Of interest, our study species crabs, cockles and mussels are of ecological importance by functioning as mobile scavengers and ecosystem engineers. Besides that, they are preyed upon by a wide variety of mobile predator species, which might have possible consequences for further disease transmissions. The disease dynamics of oyster pathogens are still poorly understood and therefore it is hard to forecast what effects it might have on higher trophic levels and spatial

extensions. However it is high likely that these mechanisms significantly affect the ecosystem functioning in different ways: losses throughout the food web, and potential changes in community structure. Ecosystem recovery is often very slow and the potential for ecosystem disruption might result in collapse and disruption. In addition, these changes are occurring in the context of additional stressors such as a changing climate, and to predict these changes require more modelling of future impacts. It also poses the question of how the host pathogen or the host:pathogen interactions might be impacted in a changing environment.

A main aim of this study was to enhance and support the Irish Pacific oyster industry, with a focus on development of new management tools. Husbandry techniques will need to change to respond to these stressors and ultimately farmers may need to depend on resistant species or there may be some changes in the species cultured. Results suggested that environmental conditions affect oyster families and oyster age classes in different ways and confirm the complexity of the host:pathogen:environment interactions. Prevalence of infection was not solely an indication of good or bad oyster performance.

Overall, results indicated that “Resistant” French stock performed better in survival and growth better than “Naïve” Irish stock, “Resistant” French half grown had better growth at high shore compared to lower shore. Obviously, the genetics of the species will influence its response to pathogens with significant better performance of “Resitant” bred species. Also, this research emphasises that movement restrictions to prevent the spread of these and other pathogens are essential due to their persistence and their ability to respond and proliferate in the environments they find themselves in. Oyster farmers at our study site supposed to transport older oysters along the shore; from high shore at their early life stage up to lowest shore when reaching consumption size. Therefore, results of the study suggest it would be worthwile to place the half-grown oysters higher up shore.

Previous studies indicated that exposure of pathogens to oysters in early life stages has beneficial effects on survival and growth when survivors at later life stage are exposed to pathogens again. This makes it worthwhile investigating the effects of subtidal and intertidal treatment before relaying of the oysters in the field. These management tools would be easy to control and implement at oyster culture sites.

The research also indicates that algae derived sulphated polysaccharides might ameliorate these infections but more information and research is needed on identifying these compounds, understanding their mode of operation, understanding in more detail aspects of the host response and how it is modulated and how effective or not they are under a range of conditions. To gain better understanding of immune stimulating activities by seaweed species (sulphated polysaccharides) and/or commercial products containing immunodulating marine sulphated polysaccharides I recommend to test for immune repsonses in a shorter time frame after exposure (3 hours, 12 hours, 24 hours). In addition, it would be worthwhile to test not only lysozyme but also immune response like; haemocytic cell counts, neutral red retention assays, nitric oxide production and expression of immunerelated genes. Besides, future studies could test other seaweed species, different seaweed concentrations, exposure to natural conditions (quality light) and test commercial algae derived products on virus and also *Vibrio aestuarianus* infected oysters.
These results however are a start to the development of new management tools and culture practises to boost the immune system of Pacific oysters to overcome times of stress.

In conclusion, results of this study are a starting point to gain a better understanding of the dynamics of Pacific oyster pathogens and diseases and could help to develop new husbandry tools to enhance and support the growth of the sector in the near future.

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Conferences: Poster and oral presentations

- Oral presentation "The role of intertidal mobile predator and scavenger shore crab *Carcinus maenas* in transmission dynamics of Pacific oyster pathogen ostreid herpesvirus-1 microVar" at Annual Meeting National Shellfish Association (NSA) on 18-22nd of March 2018 in Seattle (USA). BE Bookelaar (First author), AJ O'Reilly, SA Lynch and SC Culloty.

- Poster presentation "The 'pathogen-host-environment interplay' of oyster pathogens in the marine environment - Supporting the health of the Irish oyster industry to provide sustainability and growth of the sector" at Annual Meeting National Shellfish Association (NSA) on 18-22nd of March 2018 in Seattle (USA). BE Bookelaar (First author), SA Lynch and SC Culloty.

- Poster presentation "The effects of seaweed species *Fucus vesiculosus* and *Mastocarpus stellatus* on Pacific oyster *Crassostrea gigas* performance and pathogen development" at Annual Meeting National Shellfish Association (NSA) on 18-22nd of March 2018 in Seattle (USA). BE Bookelaar (First author), SA Lynch and SC Culloty.

- Oral presentation "Rescuing Dying Oysters?" at Science for All competition on 8 March 2018 in UCC, Cork (Ireland). BE Bookelaar

- Poster presentation "The 'pathogen-host-environment interplay' of oyster pathogens in the marine environment - Supporting the health of the Irish oyster industry to provide sustainability and growth of the sector" at Invading Pacific oysters: good, bad or ugly? International workshop on the impacts of Pacific oysters on marine ecosystems on 1-2 November 2017 at NIOZ Royal Netherlands Institute for Sea Research Texel (The Netherlands**).** BE Bookelaar (First author), SA Lynch and SC Culloty.

- Oral presentation "The 'pathogen-host-environment interplay': The interaction between OsHV-1 µVar, cohabiting invertebrates and the Pacific oyster *Crassostrea gigas*." By Ms Babette Bookelaar (UCC) at NRL 2017 on

28-30th March 2017 at the Marine Institute, Oranmore (Ireland). BE Bookelaar (First author), SA Lynch and SC Culloty.

- Oral presentation "Environment : Host : Pathogen interplay Reducing the impact of pathogens and disease in the Pacific oyster *Crassostrea gigas*" at SIP 2016 the International Congress on Invertebrate Pathology and Microbial Control on 24-28th of July 2016 in Tours (France). BE Bookelaar (First author), A Brown, SA Lynch and SC Culloty.

- Oral presentation "Environment : Host : Pathogen Reducing the impact of pathogens and disease in the Pacific oyster *Crassostrea gigas*" at Environ 2016 on 22-24th of March 2016 at University of Limerick (Ireland). BE Bookelaar (First author), A Brown, SA Lynch and SC Culloty.

- Poster presentation "Ecology and behaviour of shore crabs *Carcinus maenas* at man-made habitat and natural habitat") at Environ 2016 on 22-24th of March 2016 at University of Limerick (Ireland). BE Bookelaar (First author), AJ O'Reilly, SA Lynch and SC Culloty.