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**National University of Ireland, Cork**




# The impact of UV radiation on the health and pathogen development of the Pacific oyster (*Crassostrea gigas*)

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

School of Biological, Earth and Environmental Sciences

07/10/2022

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## 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 and intellectual property.

Gary Kett, BSc.

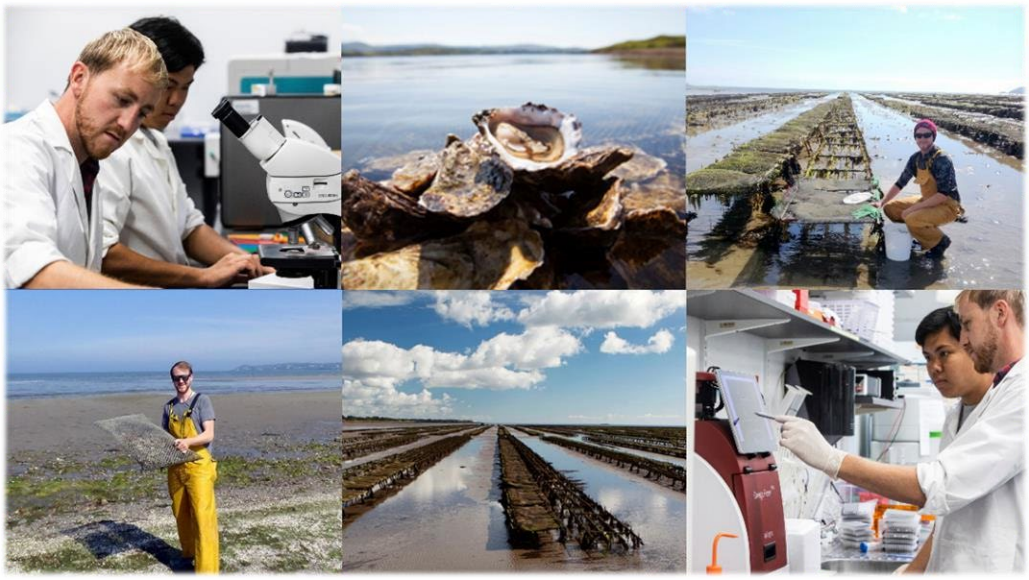


Figure *i*. A glimpse into the some of the research processes undertaken in pursuit of this doctoral thesis.

*This thesis is dedicated to my mother, Joanna Kett (née Cliffe).*

*I could not have completed this task were it not for your guidance, strength, perseverance, and humour, and for the fact you made me promise I would.*

## General abstract

Pacific oysters *Crassostrea gigas* are cultured worldwide and play an important role in global food supply and the sustainable blue economy. Oyster culture sites in Europe, USA, Australasia, and Asia have been experiencing episodic summer mass mortality events. These mortality events can be severely damaging with significant impacts on stock reliability and profitability. Summer mass mortality events are believed to have a multifactorial aetiology driven by high water temperatures and the presence of pathogens, particularly Ostreid herpesvirus-1 and variants (OsHV-1 µVar) and bacteria of the genus *Vibrio* such as *V. aestuarianus*. UV radiation (UVR)

is an intertidal stressor which functions as an ecosystem regulator. UVR has disinfectant properties with the energetic potential to damage nucleic acids of microbes inhabiting surface waters. UVR can also have both positive and negative impacts on animal immune functioning by the activation or inactivation of certain biochemical pathways. Climate model predictions show UV levels changing globally due to changes in cloud cover, aerosols, ozone, and precipitation patterns. This study aimed to investigate the impact of UV radiation (UVR) on oyster health and pathogen performance.

Firstly, a desk-based literature review study found that UVR predominantly hinders pathogens, although with varying efficacy, has mixed effects on aquatic invertebrates and has mixed effects on host-pathogen relationships. A clear knowledge gap was identified in that no study could be found which investigated the impact of UVR on bivalve health and survival. *Vibrio* bacteria are reported to be highly sensitive to UVR while herpesviruses either have high tolerance or can even be activated by solar UVR. UVR can be additive, synergistic, antagonistic, or neutral in outcomes of host-pathogen dynamics.

Secondly, novel diagnostic methods for the detection and localisation of *Vibrio* bacteria within oyster tissues were designed, a generic conventional polymerase chain reaction PCR and a DIG-labelled *in situ* hybridisation (ISH) assay. These tools were designed to complement existing PCR and qPCR tools and allow for improved understanding of pathogen behaviour inside a *C. gigas* host exposed to UVR. Primers (VibF3/VibR3) were designed to amplify a 286 bp product from the 16S ribosomal RNA gene common to all

*Vibrio* spp. and to form the ISH probe. ISH was carried out on *C. gigas* seed sourced from a *V. aestuarianus* endemic bay (n = 17) and on *C. gigas* juveniles sourced from a *V. aestuarianus* naive site (n = 12). Positive ISH signals were observed in PCR and qPCR positive *C. gigas* while no ISH signal was observed in uninfected samples from the naïve site. Direct Sangar sequencing of PCR products (n = 30), Blastn analysis and Clustal Omega alignments were used to confirm *Vibrio* sp. detection and assess similarity.

Next, to examine the effect of supplemental UV-B on *C. gigas* seed, a set of laboratory-based experiments were constructed. Various size classes of *C. gigas* seed were exposed to two conditions: i) a short duration, high intensity UV-B exposure while immersed underwater or ii) a longer duration, low intensity UV-B exposure while emersed out of water. These experimental conditions were chosen to mimic tidal immersion and emersion. The intensity of exposure was lowered in the second trial in order to carry out the treatment over the length of a typical solar peak (midday) during low tide, with the total dose typical of what would be experienced in the south coast of Ireland on a clear summer day. The impact of UV exposure on oyster health was measured by monitoring survival daily, gill tissue DNA samples were used to monitor pathogen prevalence and intensity, and histological tissue cross-sections were examined for pathological damage. Results showed that UV-B exposure negatively impacted oyster survival, most notable in the smallest seed, reducing survival by up to 35%. UV-B also impeded the development of *V. aestuarianus*, although most effects were transitory and returned to preexposure infection levels within 1 - 3 days. Moribund oysters exposed to

UVB had significantly weaker *V. aestuarianus* infection intensities than moribund oysters in the control group. OsHV-1 µVar was not detected in any sample throughout the experiment. These findings indicate that oyster mortality was caused by UV-B exposure rather than by pathogen infection. These data are the first reported impacts of UV-B on *C. gigas* health and the host-pathogen dynamic with *V. aestuarianus.* Results from this study suggest that UVR is likely to be a causative factor in *C. gigas* summer mass mortality episodes.

Lastly, to bridge the prior findings to the natural environment, a field trial was designed on a commercial oyster culture cite to investigate the impact of shore grow-out height and the resulting emersion conditions including solar UVR on *C. gigas* and pathogen performance. Emersion has been shown to have mixed effects on *C. gigas* performance, though little is known about the impact of UVR in this host-pathogen-environment model. The field experiment in this study was carried out over 5 months, in July *C. gigas* seed (n = 570) were relayed in 6 replicate mesh bags split across two shore heights equating to a +4-hour emersion time in High Shore (HS) groups compared to the Low Shore (LS) cohort. Mortality (%) was counted in the field and samples (n = 30/shore height) were returned to the lab for pathogen screening for OsHV-1 µVar and *V. aestuarianus* using PCR and qPCR. Increased oyster mortality was associated with emersion, particularly in periods of high UV exposure (>2.4 kJ/m$^2$) and high air temperatures (>21 $^o$C). Pathogen partitioning was observed, OsHV-1 µVar was detected more in high shore

cohorts while a higher prevalence of *V. aestuarianus* was detected in low shore *C. gigas*. Results indicate that environmental conditions impacted spat survival more so than pathogen infection. These findings further demonstrate that oyster mortality and infection levels are influenced by shore height and emersion time. Results from this study can be applied in husbandry practices to reduce losses during summer mass mortality events.

Research outcomes are discussed in terms of the wider framework of theoretical knowledge and global development goals, future research questions are posed and recommendations for experimental design are offered. In terms of commercial application, specific husbandry practices are suggested based on the findings of this study, however additional research should be carried out to support or improve upon these recommendations.

# Chapter 1: Introduction

## 1.1 Pacific Oyster (*Crassostrea gigas*)

### 1.1.1 Description

The Pacific oyster, *Crassostrea gigas,* is an inter- and sub-tidal marine bivalve mollusc. The elongate shell varies in colour but is generally blue grey with purple and white markings and highly fluted edges. The shell has dissimilar valves, with a flat right valve and a cupped or concave left valve.

The interior of the shell is usually pearly-white nacre material (Harris, 2008).

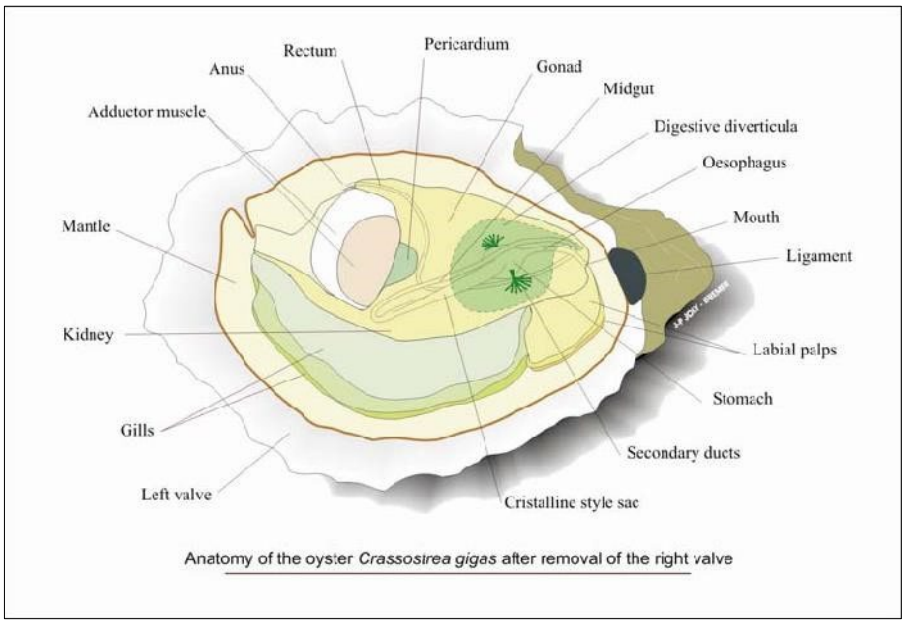



Figure 1. Anatomy of the Pacific oyster (*Crassostrea gigas*). Source: J-P. Joly, Ifremer.

### 1.1.2 Nomenclature

The genus *Crassostrea* (Sacco, 1897) contains a number of important commercial species including but not limited to the: Pacific oyster *C. gigas,* the Portuguese oyster *C. angulata* which is cultivated in Spain and Portugal (Arakawa, 1990), the Slipper cupped oyster *C. iredalei* which is cultivated in Malaysia and the Philippines (FAO, 2018), and the American (Eastern) cupped oyster *C. virginica* which is grown in the United States and in Mexico (FAO, 2018,). Recently, it has been proposed that the *Crassostrea* genus be limited to the Atlantic species only and that the Pacific species are assigned to the genera *Talonostrea* and *Magallana* based on biogeographic and phylogenetic taxonomy (Salvi *et al*. 2014). However, this designation is contested due to the long-standing history and framework of the *C. gigas* nomenclature used by oyster growers and biologists alike, amongst other reasons (Bayne *et al*., 2017, Backeljau, 2018). For the purpose of this thesis, the original and familiar genus of *Crassostrea* will be used instead of the

alternative *Magallana gigas* for applicability and relevance to industry, stakeholders, and academics.

### 1.1.3 Biology of *Crassostrea gigas*

#### *1.1.3.1 Life cycle*

*C. gigas* are protandrous hermaphrodites, initially developing as males and maturing into either males or females after a single year (Harris, 2008). Environmental variability and food abundance has been known to disrupt the sex development ratio in oysters - in areas of high food abundance, protandry is more common while in areas of food scarcity, protogyny tends to be more common (Quayle, 1969). *C. gigas* is one of the fastest growing oyster species, able to reach 15 cm after 2 - 4 years (Harris, 2008). When sexually mature, *C. gigas* is highly fecund, spawning is synchronous and generally occurs annually when waters reach temperatures of 16 - 20 $^{o}$C (Ruiz *et al*., 1992). In a single spawning event, females can produce 50 - 100 million eggs which are fertilised by sperm in the water column. The fertilised eggs develop into a veliger with the growth of a shell and then a pediveliger after development of a foot structure. The pediveliger settles on suitable substrate and is subsequently known as a spat (Quayle & Newkirk, 1986) (figure 2). This development process takes up to 30 days before spat can settle on substrate. These early life stages may be particularly vulnerable to stressors such as increased ocean acidification (Barton *et al*., 2012), warming water and incident light or photoperiod (Fabioux *et al*., 2005). Larval settlement is particularly affected by light with responses dependent on wavelength and intensity. Near UV (NUV: 375 nm) has been shown to reduce *C. gigas* larval

settlement while near infrared (NIF: 735 nm) has a positive effect on larval settlement (Kim *et al*., 2021).

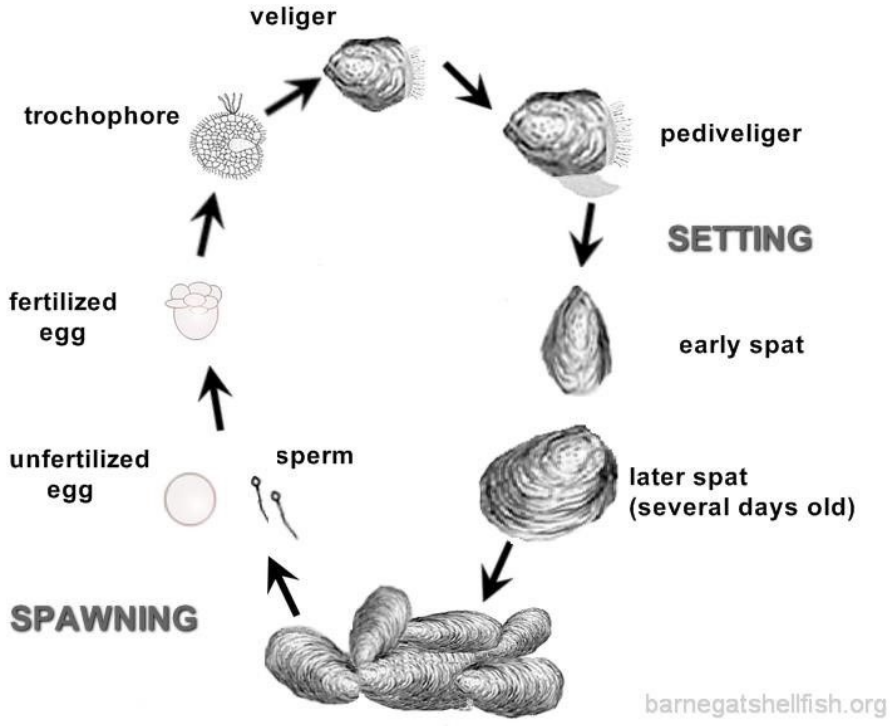


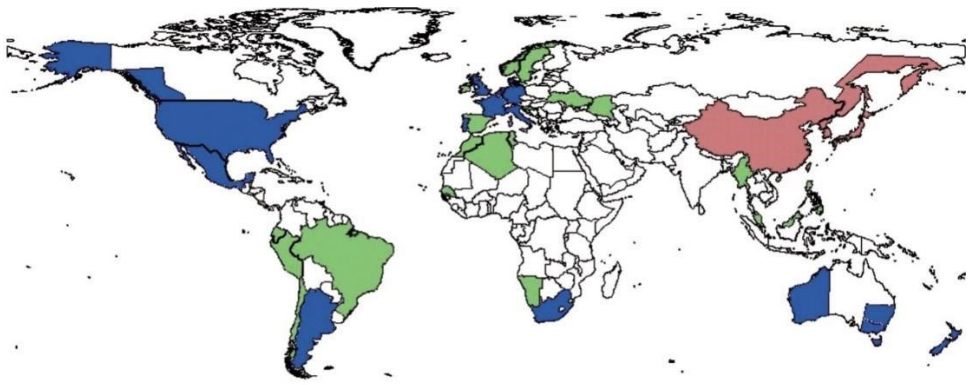

Figure 2. Life cycle of the Pacific oyster *Crassostrea gigas* source: barnegatshellfish.org.

### *1.1.3.2 Immune system*

Bivalve molluscs have an innate immune-defence system composed of cellular and humoral components. Unlike mammals and higher vertebrates, bivalves do not have the capability to develop acquired immune functions (Bachére *et al.*, 1995). However, new research in oyster genomes and the complex array of immune receptors suggest that bivalve immune responses can be quite adaptable allowing them to thrive as filter-feeders in such microbe-rich habitats (Guo *et al*., 2015). Haemocyte cells are responsible for

the cellular defence functions, while the haemolymph is the humoral component, circulating haemocytes and fluids through an open vascular system (Wang *et al*., 2018). Haemocytes can be classified into two main groups: granulocytes and agranulocytes (hyalinocytes) based on the presence or absence, respectively, of granules in the cytoplasm (Bachére *et al*., 2004). The humoral element of bivalve immune systems involves the use of lysozymes - enzymes responsible for the breakdown of bacterial cell walls (Hanington *et al*., 2010). These enzymes play an important role, not only in the innate immune system but also in digestion , anti-inflammatory responses, and growth stimulation (Pila *et al*., 2016). Haemocytes also produce Reactive Oxidative Species (ROS), particularly as a stress-response to defend against non-self particles such as pathogens, although ROS can also induce oxidative damage to oyster tissues (Donaghy *et al*., 2012, Khan *et al*., 2019).

### 1.1.4 Introductions of the Pacific oyster



Ruesink JL, et al. 2005.
Annu. Rev. Ecol. Evol. Syst. 36:643–89

Figure 3. Global distribution of Pacific oyster *C. gigas* with the introduction status colour coded. Blue: Known or highly likely to be established, Green: not established outside of aquaculture or status unknown, Red: Native range. Source: Ruesink *et al*., (2005).

Pacific oysters are native to the eastern Pacific coast of Japan, Korea, and China. North America imported the species for intensive farming to compensate for the diminishing production of native Olympia oyster (*Ostreola conchaphila)* on the west coast of America in the late 1800s and that of the eastern oyster (*Crassostrea virginica*) on the eastern US coast in the early 1900s (Harris, 2008, Trimble *et al*., 2009). Similar developments were observed around the world as indigenous bivalve species were depleted due to over-fishing, disease and pressures from coastal development, and Pacific oysters were introduced to fill the economic niche (Ruesink *et al.* 2005). Replacements occurred with the Australian flat oyster (*Ostrea angasi*) (Miossec *et al*. 2009) and the European flat oyster (*Ostrea edulis*) in France, Ireland, UK, Belgium, and the Netherlands (Miossec *et al*. 2009, Tully & Clarke 2012).

In 1965 the Pacific oyster *C. gigas* was introduced to Ireland, and due to its wide tolerance to varying water temperatures and salinity, fast growth rate and relative resistance to *Bonamia ostreae*, it became the primary species of oyster cultivation throughout Ireland and other European countries (Miossec *et al*., 2009). The history and extent of *C. gigas* introductions convey the cultural and economic importance of oyster production for many countries around the world and the significance of understanding ecological

interactions, particularly those between parasite and host. Parasites have played recurrent and consequential roles at oyster production sites, and it is vital to understand how both parasites and hosts will respond to changing environmental stimuli, increased translocations of species and changes to cultivation methods.

### 1.1.5 Production & economic impact

The Pacific oyster is a significant species for commercial aquaculture on a national and global scale. The successful cultivation of this species can be ascribed to a number of factors such as, 1) a wide tolerance to abiotic stressors including salinity (surviving 2 to 50 PSU) (Miossec *et al*., 2009), temperature (-5 $^{o}$C to 30 $^{o}$C) and emersion (Hamdoun *et al*., 2003); 2) phenotypic, trophic and spatial plasticity which allows for the adjustment to new habitats and variable food sources (Bayne, 2004, Dubois *et al*., 2007, Troost, 2010); 3) rapid growth and relatively large size which appeals to a wide consumer market (Ruesink *et al,.* 2005, FAO, 2018).

Worldwide cultivation of the Pacific oyster expanded rapidly in the late 20$^{th}$ century. In 1970, global production was at 473,000 tonnes increasing to 1.2 million tonnes in 1990 and further expanding to 3.9 million tonnes by 2000 (FAO, 2018). The rate of growth slowed in the mid-2000s as coastal urbanisation and competition for resources such as coastal development space became more frequent. Most recent figures show total global aquaculture production of *Crassostrea* spp. to be over 5.1 million tonnes in 2018, making up almost 30% of total aquaculture production (FAO, 2018). The majority of *C. gigas* for consumption are cultured (97%) compared to wild caught

fisheries (3%). China produces the largest volume (3.8 million tonnes) at 84% of total world production, with Japan (261 000 tonnes), South Korea (238 000 tonnes) and France (115 000 tonnes) (FAO, 2018).

*C. gigas* comprises roughly 10% of the EU total aquaculture product weight with 142,000 tonnes worth almost 300 million euro. The largest producing countries in the EU are France, Ireland, Spain, and Portugal (BIM, 2018). In Ireland, total oyster production has been steadily increasing at a rate of 2% – 2.5% since 2000, with an annual production of 10,122 tonnes in 2018, employing over 1,300 people overall and providing a direct revenue of 44.3 million euro as of 2018 (BIM, 2018).

### 1.1.6 Culture methods of Pacific oyster

A variety of methods are employed to culture *C. gigas* oysters. Although each method has its advantages and disadvantages, environmental conditions and source of seed supply are of importance when producers are choosing which method will be used (FAO, 2018). Oysters can be reared on the sea floor (bottom culture) or in the water column (off bottom or suspended culture). The most used production method in Ireland is PVC bag and trellis off-bottom culture, although newer developments such as moored floating cages and shelved baskets are increasingly used (BIM, 2018).

*Bottom culture*

Bottom culture, commonly used in North America and southeast Asia, involves growing seed on hard inter-tidal or sub-tidal seafloor. Often a fence or net is used to prevent predation and no other manual input is necessary

until harvest (FAO, 2018). The bottom culture technique appeals to producers as it incurs less cost in set-up and maintenance compared to off-bottom culturing, it can also be less labour intensive and more visually appealing (Walton *et al*., 2013). However, this method also incurs a higher rate of mortality from predators and anti-predation control methods often face resistance from the public (Walton *et al*., 2013).

*Off-bottom culture*

In off-bottom methods, oysters are contained within mesh grow-out bags or plastic trays, which are attached by rope or rubber bands to raised trellises on the intertidal shore or suspended from lines or racks.

- Bag and trellis method (figure 4): Oysters are contained within mesh bags which are secured onto horizontal metal racks placed out throughout the grow site. This method is highly suitable in shallow bays with a large tidal range as regular access is required to maintain

  the framework (BIM, 2019). Some advantages of this method include reduced predation and siltation, increased efficiency of space using three dimensions of the water column and reduced expense of harvesting (Walton *et al*., 2013). However, the bag and trellis method is more expensive and labour intensive in the initial set-up and maintenance, and may face opposition due to visual impact and restrictions on boating and fishing (Walton *et al*., 2013).

- Suspended culture or floating culture: Like the bag and trellis method, oysters are contained within mesh bags, which are supported in the water column by moored floating buoys and move with the flow and ebb of the tide. This facilitates continuous movement and flipping of the bags and makes use of the three dimensions of the water column, however this method can be very labour intensive and visually unappealing (Walton *et al*., 2013).



Figure 4. Off-bottom bag and trellis oyster aquaculture in Dungarvan Bay, Co. Waterford (Photo: Gary Kett)

### 1.1.7 Pathogens and disease of the Pacific oyster *C. gigas*

Microbes are vastly abundant and important in the marine environment. The total number of marine bacterial and archaeal cells $ml^{-1}$ in the open ocean is estimated to be between $10^{28}$ - $10^{29}$ (Suttle & Chen, 1992, Danvoro *et al*., 2011), almost equal to the estimated number of viruses at $10^{30}$ $m^{-1}$ (Suttle, 2005). Marine microbes affect global oceanic productivity, biogeochemistry, and recycling of nutrients, matter, and energy (Salazar & Sunagawa, 2017). Pathogenic microbes are those which can infect hosts and cause disease

dependent on the virulence of organisms (Casadevall, 2017). Pathogenic microbes can have serious implications for wild and farmed animal populations and consequently human health and food security (De Schryver & Vadstein, 2014). In bivalve aquaculture, pathogenic microbes typically include bacteria, viruses, protistans, as well as some fungal and helminth parasites (Zanella *et al*., 2017). Disease occurrence in farmed bivalves is influenced by a range of factors including environmental conditions, husbandry practices, natural and selected stock resistance, and spatial and temporal epidemiology (Pernet *et al*., 2016). Managing disease outbreak risk is a complex task requiring extensive knowledge of host-pathogen ecology, transmission dynamics and the regulatory framework which governs the industry.

#### *1.1.7.1 Vibrio aestuarianus*

The genus *Vibrio* is a ubiquitous and abundant species of gram-negative, motile, straight, or curved, rod-shaped marine bacteria. They are especially found in association with marine and estuarine organisms including plankton (Heidelberg *et al*., 2002), corals (Ben-Haim & Rosenberg, 2002) and fish (Ringo *et al*., 2010) and molluscs (Romalde *et al*., 2014). Optimal growth conditions of *Vibrio* species include temperatures greater than 15º C and salinities less than 25 PSU (Baker-Austin *et al*., 2010). *Vibrio* is a highly diverse genus containing more than 100 species, at least 50 of which were described for the first time in the past 15 years due to new molecular techniques of species identification (Romalde & Barja, 2010). Some *Vibrio* species are known to be important for ecological systems such as the carbon cycle and osmoregulation (Johnson, 2013). However, several species are

zoonotic or waterborne vectors of disease in humans e.g. *V. cholerae*, the etiological agent of cholera, and *V. vulnificus* and *V. parahaemolyticus* are known to cause non-cholera *Vibrio* infections in humans (Volety *et al*., 1999, Almagro-Moreno & Taylor, 2013). Many *Vibrio* species are pathogenic in the marine ecosystem causing a wide range of diseases, particularly in bivalve molluscs, *Vibrio* species known to cause disease in bivalves include *V. vulnificus*, *V. parahaemolyticus*, *V. tapetis*, *V. splendidus, V. harveyi*, *V. mytili*, *V. pectenicida, V. alginolyticus* and *V. aesturianus* (Romalde *et al*., 2014). Recent research suggest that *Vibrio* species as infectious etiological agents are likely to increase in numbers with warming ocean water. (Paillard *et al*., 2004, Vezulli *et al*., 2013, Lupo *et al*., 2020). Indeed, *Vibrio* outbreaks have been strongly associated with warming in temperate regions such as Peru (Martinez-Urtaza et al., 2008), Chile (Gonzalez-Escalona et al., 2005), and across Europe (Baker-Austin et al., 2010, Rowley *et al*., 2014, EFSA, 2015). These increasing temperatures would have the compounding effect of decreased or compromised immunity in bivalves (King *et al*., 2019, Rahman *et al*, 2019). Furthermore, changing temperature has also been shown to affect other aspects of the oyster microbiome which plays a role in infection susceptibility and immune response (Notaro *et al*., 2021, Dugeny *et al*, 2022).

*Vibrio aesturianus* (formerly named *Vibrio aestuarii*) was first described by Tilson & Seidler (1983) as occurring in oysters and clams in the west coast of North America and has recently been associated with outbreaks of the "summer mortality syndrome" in oyster production sites in France (Gay *et al*., 2004) and Ireland causing mass mortalities. *V. aestuarianus* was first detected in Ireland in *C. gigas* samples collected between 2008 to 2012, across all host

age groups reaching 100% in prevalence in some groups and often coinciding with OsHV-1 infection (EFSA, 2015). The role of *Vibrio* species e.g. *V. splendidus* in oyster mortality has been identified in France (Lacoste *et al.,* 2001,, Gay *et al.*, 2004) *and V. aestuarianus* is commonly associated with increased oyster mortality in Ireland (Axén *et al*., 2019). Drivers of these mass mortality events are thought to be multifactorial involving high temperatures, and sub-optimal environmental and

physiological conditions (Garnier *et al*., 2007, Green *et al*., 2016), and are often polymicrobial involving co-infections by multiple pathogens (Petton *et al*., 2015).

#### *1.1.7.2 Ostreid herpesvirus - 1*

Ostreid herpesvirus-1 (OsHV-1) and variants, in particular ostreid herpesvirus-1 microVar (OsHV-1 µVar), is a double stranded DNA virus, which has been associated with significant mortalities in *C. gigas* and other bivalve species including the flat oyster *O. edulis*, the Palourde clam *Ruditapes decussatus*, the Manila clam *R. philippinarum* and the King scallop *Pecten maximus* (www.eurl-mollusc.eu). It is the only described species in the Genus *Ostreavirus*, Family Malacoherpesviridae in the Order *Herpesvirales*. OsHV-1 has a linear double-stranded DNA encapsulated by a spherical pleomorphic enveloped icosahedral T=16 capsid. The genetic sequence of OsHV-1 was first described in 2005 and has an estimated length of 207 Kbp (Davidson *et al.*, 2005). Numerous studies have suggested OsHV1 as the causative agent in the mass summer mortalities observed throughout Europe which can result in reduction of stocks of 50-100% (Renault *et al*.,

2000, Arzul *et al*., 2001, Friedman *et al*., 2005, Prado-Alvarez *et al*., 2016). Mortality in OsHV-1 (Var) infected oysters is affected by numerous biotic and abiotic factors such as physiological and reproductive state (Cotter *et al*., 2010), age and growth rate (Pernet *et al*., 2012), farming practices (Pernet *et al*., 2012), co-infections with other pathogens such as *Vibrio* bacteria (Petton *et al*., 2015), as well as environmental conditions such as water temperature, quality, and salinity (Samain & McCombie, 2008, Malham *et al*., 2009, Pernet *et al*., 2012).

A more recent variant genotype, ostreid herpes virus-1 microVar (OsHV-1 µVar), is believed to be a more virulent variant of OsHV-1. The variant was first isolated in 2008 and was immediately associated with mass summer mortalities of Pacific oysters in France (Segarra *et al*., 2010), and subsequently in Ireland (Renault *et al*. 2000, Lynch *et al*, 2012, Peeler *et al*., 2012), the UK (OIE, 2014), and the Adriatic Sea (Burioli *et al*., 2016) as well as Australia (Jenkins *et al*., 2013, Paul-Pont *et al*., 2013) and New Zealand (OIE, 2014). OsHV-1 µVar is distinguished from the reference type (OsHV1) by a 2.8 Kbp deletion (Renault & Arzul, 2001) and an insertion of 27 bp along with other polymorphisms (Segarra *et* al., 2010, Mandas & Salati, 2017). OsHV-1 µVar also differs from the reference type by exhibiting a genetic fragment length of 529 bp, defined by systematic deletion of 12-13 bp in the TAC region between ORF4 and ORF5 (Mandas & Salati, 2017).

## 1.2 Climate and environmental parameters

### 1.2.1 Ozone layer depletion

The ozone layer blocks biologically harmful ultraviolet light rays from reaching Earth (WMO, 2011). However, historic emissions of chlorofluorocarbons (CFCs) and other halogenated substances from anthropogenic activity have depleted or thinned the ozone layer (Rowland, 2006, WMO, 2011). Ozone depletion sparked worldwide concern as increasing levels of UV-B could pass through the ozone layer and have deleterious effects to biological systems on Earth (Caldwell *et* al., 1998, Whitehead *et al*., 2000). This led to the creation of international treaties such as the Montreal Protocol on Substances that Deplete the Ozone Layer, the Vienna Convention for the Protection of the Ozone Layer and the Climate and Clean Air Coalition (EEAP, 2019). In the northern hemisphere, ozone depletion remains high, and ozone reached a record low in 2011 (Manney *et al*., 2011). On a global scale, ozone depletion was most severe during the 1980s and early 1990s (WMO, 2011). More recent and regional data show that stratospheric ozone levels in the region 35° N – 60° N i.e., southern Mediterranean to southern Scandinavia, between 2008 – 2011 were reduced by 6% of that which they were in the pre-1980s period. Recent comprehensive research by the United Nations Environment Program, specifically the Environmental Effects Assessment Panel for the Parties to the Montreal Protocol, released an assessment of concurrent literature. This review summarised that the recent and predicted increases in UV-B are largely linked to climate change, i.e. variations in cloud cover, ice and snow presence and

atmospheric aerosols, rather than ozone concentrations (EEAP, 2019). The latest report from the UNEP has acknowledged that changing weather patterns are likely to play an important role in host-pathogen interactions in aquatic environments as increased precipitation and resulting organic matter (DOM) from terrestrial run-off, offers protection to water-borne pathogens from disinfecting solar UV radiation. This increase in pathogens and disease may have implications for human and animal health, aquaculture, and food security (Williamson *et al*., 2017, Barnes *et al*., 2022).

### 1.2.2 Ultraviolet (UV) radiation.

Solar ultraviolet (UV) radiation is categorised into three primary subtypes defined by their wavelength on the electromagnetic spectrum. Longer wavelengths such as UV-A are less phototoxic but important in animal vision and plant-animal communication (Aphalo *et al*., 2012). Medium and short wavelengths such as UV-B, and UV-C are more photochemically reactive and induce a wide range of cytotoxic and genetic effects in aquatic animals (Vincent & Neale, 2000). Short wavelengths carry more energetic potential and thus can induce more genetic damage, however, UV-C does not penetrate the atmosphere so is of little ecological concern in the natural environment (Hader & Sinha, 2005, Rastogi *et al*., 2010, Aphalo *et al*., 2012). The UV-B wave band of 280 - 315 nm consists of less than 0.8% of the electromagnetic energy to reach the Earth's crust, the remainder being UV-A and longer waves such as visible light. Yet, it is still responsible for roughly half of the photochemical UV effects in aquatic environments (Whitehead *et al*., 2000). To understand the effects of UV radiation on marine organisms, it is vital to

understand the physical and chemical properties of the water to assess penetration and attenuation of the light waves (Diaz *et al*., 2000, Tedetti & Sempere, 2006). As solar radiation passes through a water body, its spectrum changes, and shorter wavelengths in particular will be rapidly attenuated (Aphalo *et al*., 2012). The main physical characteristics of water which control the attenuation of UV radiation are absorption and scattering (Whitehead *et al*., 2000, Tedetti & Sempere, 2006). These factors are controlled by water constituents, primarily chromophoric (coloured) dissolved organic matter (CDOM) and dissolved organic carbon (DOC).

## 1.3 UV-B induced DNA damage

The most significant deleterious biochemical alteration caused by UV radiation is the structural modification of DNA (Sancar *et al*., 2004, Rastogi *et al*., 2010). The most common mutagenic lesions in aquatic organisms are cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine-pyrimidone adduct (6-4 PPs) and their resulting Dewar isomers (Hader & Sinha, 2005). Some organisms have the capacity to adapt to UV-B radiation through behavioural responses, physical or chemical screening, quenching, or repairing the inflicted damage (Buma *et al*., 2003, Takahashi & Ohnishi, 2009). Behavioural responses such as movement and avoidance are generally the first level of defence against harmful radiation (Buma *et al*., 2003). However, sessile benthic organisms such as oysters rely on screening (i.e. reflection and absorption to protect sensitive internal tissues), quenching (use of antioxidants to reduce UV damage) and repair (light or dark mediated pathways) (Buma *et al*., 2003).

The rate of DNA damage accumulation and subsequent repair is highly dependent on depth of the organism (Boelen *et al*., 2001, Banaszack, 2007), intertidal habitat position (Lyons *et al*., 1998), motility (Buma *et al*., 2003), and time of day (Jeffery *et al*., 1996, Weinbauer *et al*., 1999). It is worth noting that sessile intertidal organisms in the intertidal zone or in the upper surface of shallow water may have prolonged exposure to UV-B radiation if water levels do not rise enough to attenuate UV-B (Buma *et al*., 2003).

## 1.4 Aim and research questions

This thesis aims to examine the dynamics of host-pathogen relationships under the influence of UV radiation in an aquatic habitat. The Pacific oyster *Crassostrea gigas* is used as a model species due to its global commercial and ecological significance. The *C. gigas* culture industry increasingly experiences mass mortality events which are largely driven by environmental and pathogenic drivers. Two pathogens were chosen for this study, *Vibrio aestuarianus* bacteria and Ostreid herpesvirus-1 µVar, as they have been identified as causative agents in oyster mortality events (Petton *et al*., 2015). The impact of UV radiation is explored in terms of oyster health and survival and pathogen development by developing the following research questions:

1) What do we currently know about the impact of UV radiation on bivalve species and aquatic pathogens, can we use that information to hypothesise on the effect UV, particularly UV-B will have on *C. gigas*, *V. aestuarianus*, and Osterid herpesvirus-1 (OsHV-1 µVar)

   a. What is the existing information on the impact of UV radiation on bivalve species and aquatic pathogens?

b. Does the literature and research to date show a consistent pattern of UV impacts on hosts, pathogens, and their interactions?

c. Can we use this information to hypothesise on the impact UVB will have on *C. gigas*, *V. aestuarianus*, and Osterid herpesvirus-1 (OsHV-1 µVar) under a variety of husbandry scenarios?

2) Can the diagnostic arsenal of *Vibrio* infections in bivalve shellfish be expanded upon to improve our understanding of *V. aestuarianus* infection in *C. gigas*.

a. In Vibrio infections, where within *C. gigas* tissues are *the bacteria* detected? This is investigated to better understand the behaviour of bacteria in the host, particularly under UV stress if bacteria migrate to more internal tissues.

b. Is it possible to determine if low *Vibrio* PCR detections are the result of fewer viable bacteria or the result of bacteria not being detected by the PCR method? This is important so to ensure the validity of a commonly applied diagnostic technique of only screening gill DNA.

c. How are the diagnostic tools that are available - histological and molecular biological best utilised to maximise detection of *Vibrio*?

3) What impact does supplemental UV-B radiation have on the health of *C. gigas* and the performance of *V. aestuarianus*?

a. Under laboratory settings what impact does artificial UV-B have on the health of *C. gigas* and the host: pathogen dynamics?
b. How do different simulated conditions – that mirror husbandry techniques impact on *Vibrio* development?

4) Does growing height influence *C. gigas* performance and pathogen partitioning?
   a. In the field, how does shore height affect *C. gigas* health and development?
   b. How do field and husbandry conditions impact on primary pathogens, *V. aestuarianus* and OsHV-1 µVar particularly in relation to emersion times?

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# Chapter 2: Solar UV radiation modulates animal health and pathogen prevalence in coastal habitats – knowledge gaps and implications for bivalve aquaculture.

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## 2.1 Abstract

Ultraviolet radiation (UVR) is an important environmental factor which can impact directly, or indirectly, on the health of organisms. UVR also has the potential to inactivate pathogens in surface waters. As a result, UVR can alter hostpathogen relationships. Bivalve species are threatened by various pathogens. Here, we assessed the impacts of UVR on i) bivalves, ii) bivalve pathogens and iii) the bivalve host–pathogen relationship. UVR consistently impedes pathogens. However, the effect of UVR on marine animals is variable with both positive and negative impacts. The limited available data allude to the potential to exploit

natural UVR for disease management in aquaculture, but also highlight a striking knowledge gap and uncertainty relating to climate change.

Keywords: UV Radiation, Bivalve, Pathogen, Host-Pathogen dynamics, Aquaculture, Solar disinfection

## 2.2 Introduction

The United Nations Environmental Effects Assessment Panel (EEAP) has identified the paucity of data surrounding solar disinfection, and its effects on animals and humans, as a major knowledge gap, and that filling this gap is crucial in maintaining health and water security into the future (Environmental Effects Assessment Panel [EEAP], 2019). Solar disinfection is the UVR mediated inactivation of pathogens resulting from the impact of ultraviolet radiation on cell structures, such as DNA, lipids, and membranes (Kohn *et al*., 2016, Williamson *et al*, 2017). Solar radiation is widely recognised as a significant germicide in surface waters (Burkhardt III, *et al*., 2000, Lytle & Sagripanti, 2005, Williamson *et al.*, 2017), and is estimated to be responsible for the inactivation of between one quarter and two thirds of virus particles in seawater (Noble & Fuhrman, 1997). Global bivalve aquaculture has a net worth of over 16 billion USD (Food and Agriculture Organisation [FAO], 2018) however, in recent years, disease outbreaks and mass mortality events have been increasing at coastal bivalve production sites globally (Elston *et al*., 2008, Samain & McCombie, 2008, Lynch *et al.*, 2012, Dubert *et al*., 2017). Disease outbreaks and mass mortality events have significant economic and social impacts on revenue and local communities

(Guillotreau *et al*, 2017, Krause *et al*., 2019). Given the important role of bivalve molluscs in sustainable food production and ecosystem functioning (FAO, 2018, van der Schatte Olivier *et al.*, 2018), and the recognised potential of UV radiation for pathogen control, it is pertinent to explore the potential impacts of UV on bivalve health and bivalve host-parasite relationships.

The ultraviolet (UV) part of the solar spectrum comprises highly reactive radiation that can be categorised according to the wavelengths as UV-A (□□□□ — □□□ nm), UV-B (□□□□ — 315 nm) and UV-C (□□□□ — □□□ nm) (EEAP, 2019). Only solar UV-A and longer UV-B wavelengths penetrate into the biosphere, although shorter UV-B wavelengths and UV-C are commonly generated by artificial light sources for applications such as disinfection (Acra *et al*., 1990). Despite the reactive characteristics of all UV wavelengths, such radiation can have both beneficial and detrimental impacts on human, animal, plant and microbial life (EEAP, 2019). Examples of positive impacts of UV include immunomodulation and vitamin D synthesis in humans (Grant, 2003, Hart *et al*., 2011). Another beneficial UV effect relates to UV vision, present in many animal species, and its impact on animal-plant relationships, foraging, communication, navigation, and mateselection (Hunt *et al*., 2001, Kevan *et al*., 2001, Honkavaara *et al*., 2002, Dyer & Chittka, 2004). Negative impacts of UV exposure in humans and animals include mutagenesis, skin cancers, photodermatoses, eye diseases and immune system suppression. In some cases, UV can induce activation of latent herpes simplex virus and human papillomavirus (Norval *et al*., 2007).

Yet, UV is also widely known as a disinfectant of bacterial, viral, fungal, and protozoan pathogens (Morris, 1972, Chang *et al*., 1985, Acra *et al*., 1990).

Penetration of UV radiation (UVR) through the water column depends on absorption and scattering, primarily caused by coloured dissolved organic matter (CDOM) and suspended particulate matter, respectively. The latter includes both biotic, e.g. phytoplankton, and abiotic matter (Scully & Lean, 1994, Nelson & Guarda, 1995, Aphalo & Albert, 2012). In turn, optical properties of water are dependent on climate characteristics such as temperature, acidification, and local circulation and mixing patterns which can all affect the levels and distribution of organic matter in the water column (Behrenfeld *et al.*, 2006, Brewin *et al.*, 2015). UV penetration depth is also dependent on the UV wavelength as shorter wavelengths are attenuated more rapidly, thus UV-A travels further through the water column than UV-B which is usually attenuated in the upper photic layer (Smyth, 2011). Tedetti & Sempéré, (2006) provide a review on UV penetration depths in a range of marine habitats. Coastal waters have relatively low UV penetration compared with open oceans. The Z10% depth, i.e. the depth at which the surface irradiance is reduced to 10%, ranges from 0.2 to 13 meters and 0.08 to 6 meters, for UV-A (340 nm) and UV-B (305 nm), respectively, for coastal waters of Northern Europe. In comparison, in the open Atlantic Ocean the Z10% for UV-A and UV-B can be as much as 38 meters and 17 meters, respectively (Tedetti & Sempéré, 2006).

Bivalve molluscs, such as mussels, clams, scallops, cockles, abalone, and oysters, are calcifying, filter-feeding, intertidal organisms. Bivalve consumption has grown by almost 3% annually since 2000 and bivalves have been identified as an important food source to meet the growing demands of a rapidly expanding world population (FAO, 2018). Bivalves are also of great ecological significance providing a range of ecosystem services such as water filtration, denitrification, and carbon sequestration (Gallardi, 2014, van der Schatte Olivier *et al.*, 2018). Maintaining healthy bivalve populations is key to their ecosystem function, and central to sustainable culturing (FAO, 2018). At present, natural and farmed bivalve populations are challenged by a wide variety of water-borne pathogens and parasites. Bivalve molluscs can easily accumulate water-borne pathogens due to the filtration of large volumes of water (Ben-Horin *et al.*, 2015, Song *et al.*, 2016). Bivalve molluscs, like other organisms, have mechanisms of defense against UV radiation. The exterior calcium carbonate shell which is coloured with pigments such as melanin, carotenoids and tetrapyrroles, protects the soft internal tissues from the external environment, including UV radiation (Williams, 2017). Internally, bivalves contain photoprotective coumpounds such as mycosporine-like amino acids (MAAs) and carotenoids (Naoko *et al*., 2020).

Primary pathogens of interest to bivalve aquaculture in Europe are the Ostreid herpesvirus (OsHV-1) and its variants (OsHV-1 µVar) as well as bacterial species of the genus *Vibrio* e.g. *V. aestuarianus* and *V. splendidus*. These pathogens have been linked to recurring episodes of mass mortalities in Pacific oysters *Crassostrea gigas* throughout Europe (Samain & McCombie,

2008, Lynch *et al.*, 2012, Pernet *et al.*, 2014, European Food Safety Authority [EFSA], 2015, Solomieu *et al.*, 2015). Other *Vibrio* species which have been linked to disease and mortality in bivalves include *V. anguillarum, V. tubiashii,* and *V. tapetis* (Ben-Horin *et al.*, 2015, Travers *et al.*, 2015).

Common non-vibrio bacterial pathogens include species of the genera *Pseudomonas, Aeromonas* and *Nocardia*. Common viral diseases include *Iridoviridae* species including gill necrosis virus (GNV), haemocyte infection virus (HIV), and oyster velar virus (OVV) (Zannella *et al.*, 2017). The genus *Norovirus* (NoV), belonging to the Calciviridae, are particularly problematic as they can be transferred from bivalve molluscs to humans upon ingestion, which can lead to human health problems in certain cases (Bartsch *et al*., 2016, Razafimahefa *et al*., 2019). Furthermore, UV-depuration of bivalves, which can reduce most bacterial contamination, is not capable of reducing NoV loads below infectious doses in most cases (McLeod *et al*., 2017).

Protozoans have also caused great damage to bivalve production sites. Oysters and clams have experienced outbreaks of species belonging to the genera *Bonamia, Marteilia, Haplosporidium* and *Perkinsus* (Culloty & Mulcahy, 2007, Engelsma *et al*., 2014, Zannella *et al.*, 2017). The losses associated with these outbreaks pose a significant threat to the global mollusc aquaculture industry, which had an estimated worth of $30 billion USD in 2016 (FAO, 2018). Outbreaks of bivalve diseases are expected to increase in coming years due to changing marine environments (Callaway *et al*., 2012, Rowley *et al*., 2014). It is therefore important to study the impacts of changing marine

environments on aquatic pathogens to better understand, predict and mitigate against such outbreaks.

In this study the relationship between marine bivalves, pathogens and UV radiation was explored (Figure 1). A systematic literature search of published data was undertaken between January 2018 and December 2019, using a combination of pertinent terms; Ultraviolet, UV, UV-A, UV-B, UV-C, Solar radiation, Health, Immuno-suppression, Immuno-modulation, Disease, Pathogen, Parasite, Bivalve, Host-Parasite interaction. Databases searched include: ScienceDirect, Web of Science, JSTOR, Scopus, Wiley Online Library and Google Scholar. The aim of the analysis presented in this paper is to 1) capture existing knowledge on the effects of UVR on bivalves, their water-borne pathogens and parasites, and the relationship between them, and 2) to explore the potential, future role of solar UV-A and UV-B in the biosecurity of commercial bivalve aquaculture. Throughout this systematic review, care was taken to find and review literature in an unbiased fasion, seeking and reporting positive, negative and neutral outcomes and not omiiting any literature that was deemed relevant.

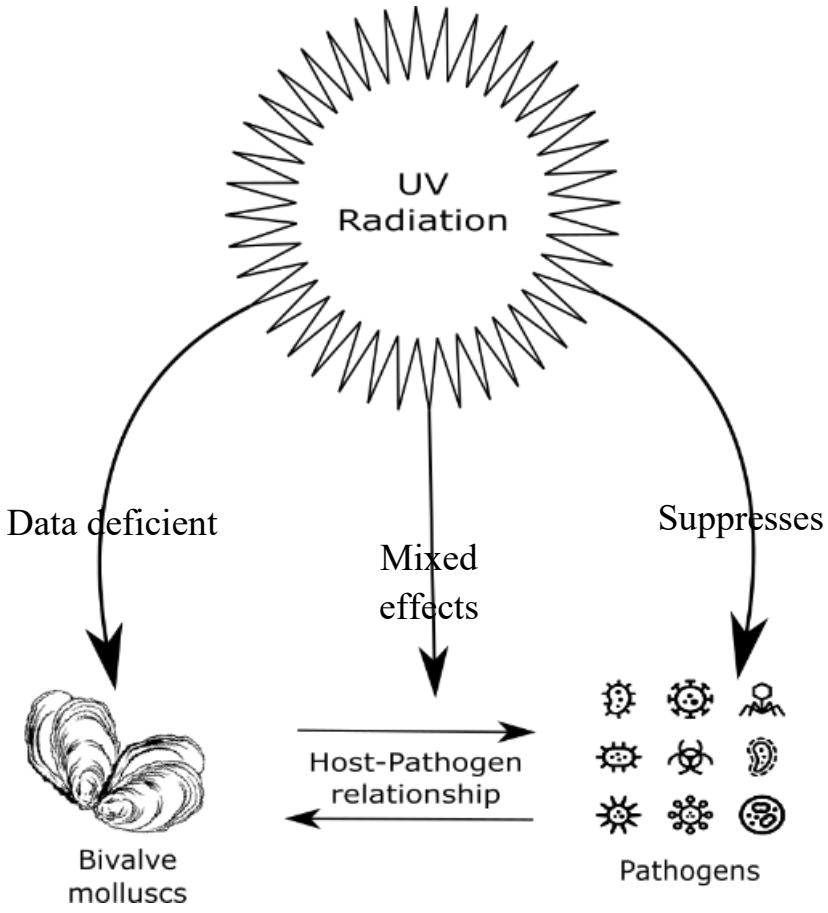



Figure 1. Schematic depicting the potential effects of UVR on host – pathogen relationships. UVR can suppress pathogens mainly through deleterious effects on DNA. UVR can also impact on molluscs, although the literature is poorly developed. Finally, UVR can modulate the immune-system of the mollusc, altering host-pathogen relationships. The net UV effect on hostpathogen relationships is highly variable and can include antagonistic, additive, or synergistic effects.

## 2.3 Effects of UV radiation on organisms and systems

### 2.3.1 UV and marine macrobiota

A large number of reviews and meta-analyses have been produced in recent years encapsulating the current reseach regarding UVR effects on aquatic biota. Here,

these reviews are discussed with reference to the findings of over 7,000 studies. There has been considerable research into the effects of UV radiation on marine organisms such as zooplankton (Williamson *et al.*, 1994), crustaceans (Rautio & Tartarotti, 2010), echinoderms (Lamare, *et al*., 2011), gastropods (Davis *et al.*, 2013), fish (Blazer *et al.*, 1997) and cetaceans (Martinez-Levasseur *et al.*, 2013). However, effects of UVR on bivalve species have not been studied to the same extent as for other animal groups. This is a knowledge gap which needs to be addressed to enhance food production and to better understand ecosystem interactions. Dahms & Lee (2010), reviewed the literature surrounding UVR impacts on marine ectotherms and concluded that UVR is a threat to natural populations as reproductive health is impaired by UVR, although defence mechanisms, avoidance, or repair can offset damage. Lamare *et al*. (2011), reviewed 65 publications regarding UVR and echinoderms and found UV induced strong avoidance responses while also boosting protective damage repair mechanisms. The review discusses different types of damage including inhibited development, fertilisation and motility in sperm, and increased mortality and DNA damage. Llabrés *et al.* (2013), reviewed 1784 experiments involving UV-B and marine biota including cnidarians, crustaceans, echinoderms, tunicates, fish and molluscs amongst others. The most common finding was increased mortality following exposure to UV-B. More recently, Jin *et al*. (2017), analysed results of 4,995 studies on the responses of marine and freshwater algal species to UV. Mortality was the most common response to increased UV-B followed by molecular and cellular alterations. Peng *et al.* (2017) analysed 146 studies on 127 species of freshwater plankton, fish and amphibians and concluded that UV-B had

a significant negative effect on metabolism, behaviour, growth, and reproduction, cellular-molecular responses and survival. The literature contains ample evidence of detrimental UV effects on marine organisms. Yet, no reference to bivalve molluscs was found in any of the aforementioned studies.

One further area where literature is lacking is in the responses of different life stages to UVR. This gap in knowledge is particularly relevant where larval, juvenile, or adult life-stages display different physiological, biochemical and/or behavioural traits. Macroalgal spores are more susceptible to UV damage than sporophytic and gametophytic structures (Roleda *et al*., 2007, Jin *et al*., 2017). Similarly, early life stages i.e. gametes and larvae, of many heterotrophs and ectotherms are more vulnerable to UV induced damage than juvenile or adults as described in multi-species meta-analyses (Bancroft *et al*., 2007, 2008, Dahm *et al*., 2011, Llabrés *et al*, 2013). However, UV-sensitivity of early-life stages is not universal, and some studies have shown the opposite (Peng *et al*., 2017, Jolkinen *et al*., 2008). It is important to be aware of variations in sensitivity and tolerance as different life stages may inhabit different spatio-temporal niches characterised by different UVR intensities (Dahms *et al*., 2011, Alves & Agustí, 2020). For example, the larval stage of some species is planktonic and inhabits the upper photic layer where UVexposure is most relevant, especially under certain environmental conditions

e.g. low phytoplankton and nutrient levels, clear cloudless skies and low ozone areas (Dahms *et al*., 2011).

Effects of UV-B exposure on marine biota may be direct, as described above, or alternatively indirect, mediated through trophic chains. For instance, UVB exposure can decrease the polyunsaturated fatty acid (PUFA) content in phytoplankton (Wang & Chai, 1994, Skerratt *et al.*, 1998) with subsequent effects on consumers. Primary producers are the main source of PUFAs in the marine environment (Guschina & Harwood, 2009, Zhukova, 2019). PUFAs are important dietary elements in vertebrates and invertebrates alike, and are essential for cell membrane function, acting as precursors to hormones and modulating immune systems (Brett & Müller-Navarra, 1997, Zhukova, 2019). PUFAs can support immune function in shellfish (Delaporte *et al*., 2003), fish (Arts & Kohler, 2009), and other vertebrates including humans (Wu & Meydani, 1998, Calder, 2014, Venugopal & Gopakumar, 2017). High PUFA diets for bivalve molluscs such as manilla clams *Ruditapes philippinarum* and Pacific oysters *Crassostrea gigas* can modulate immune function through increases in total haemocyte count (THC), percentage of granulocytes, phagocytic rate and oxidative activity (Delaporte *et al*., 2003). Moreover, haemocytes of *C. gigas* supplemented with the PUFA arachidonic acid (ArA) display lower susceptibility to the common bacterial pathogen *Vibrio aestuarianus* (Delaporte *et al.*, 2006).

### 2.3.2 UV, pathogens, and parasites.

Parasitism is often perceived as a negative biotic stress for host organisms. Yet, parasites also play an important role in regulating host populations by influencing behaviour, growth and reproductive success as well as affecting community structure and trophic interactions (Marcogliese, 2004, 2008). Nevertheless, due to the threat

posed by aquatic microbes to humans, wildlife and commercial species, the level of UV required to inactivate bacterial, viral, protozoan, and fungal pathogens has been researched in considerable detail. Studies predominately show an inhibitory effect i.e. disinfection of pathogens by UV radiation, although the extent of disinfection depends on many factors such as wavelength, dose, and pathogen biology (Morris, 1972, Liltved *et al*., 1995, Chang *et al.*, 1985, Acra *et al*., 1990, Williamson *et al.*, 2017). Shortwave UV-C (~254 nm; generated using artificial UV-lamps) is frequently used to inactivate pathogens in water, food products, crops and equipment, and aquaculture products (Gray, 2014). However, longer wave, solar UV-B and UV-A radiation may also affect parasitism either through direct or indirect exposure effects (Häder *et al*., 2015). The antimicrobial properties of solar UVR have been utilised to create cheap and effective water purification treatment (Acra *et al*., 1990, Wegelin *et al.*, 1994, Dejung *et al.*, 2007, Davies *et al.*, 2009). UV treatment, particularly using UV-C, is effective in inactivating various classes of pathogens including bacteria, viruses, protozoa and fungi (Hijnen *et al*., 2006). The mechanisms behind UV induced damage and repair of DNA, proteins, and lipids are beyond the scope of this review, but comprehensive information on these topics can be found in Sinha & Häder (2002), Buma *et al*. (2003), Hijnen *et al*. (2006).

## 2.4 Effects of UVR on bivalves, their pathogens, and host-pathogen relationships.

### 2.4.1 Effects of UVR on bivalve molluscs

A total of eight published studies on the effects of UVR on bivalve molluscs were found in the literature (Table 1). Some studies (4) explicitly identified a role of UV-B, otherwise studies showed the combined effects of UV-B, UVA and/or Photosynthetically Active Radiation (PAR) or the combined influence

of UVR, temperature and light. UV can reduce the diversity and biomass of the biofouling community, including *Mytilus edulis*, yet these effects can be transitory (Wahl *et al*., 2004). In this study, UV-A had a greater effect on decreasing biomass and diversity of shallow fouling communities than UV-B, although both together had the strongest effect. Similarly, UV exposure can reduce the surface cover of the green mussel *Perna viridis* and the horse mussel *Modiolus comptus* compared to a no-UV treatment (Dobretsov *et al*., 2005). Conversely, *M. edulis* density and recruitment can be positively correlated with UV-B and broadband UV, respectively (Lotze *et al*., 2002, Molis *et al*., 2003). With regard to dispersal and survival, Hoyer *et al.*, (2014) found no negative effect of UV on the larvae of the Asian clam *Corbicula fluminea*. At the molecular level, Regoli *et al.*, (2000), measured the Total Oxidant Scavenging Capacity (TOSC) of scallops from polar (*Adamussium colbecki, Chlamys islandicus*) and temperate (*Pecten jacobaeus*) regions. It was found that the Antarctic scallop *A. colbecki* had significantly higher TOSC compared to the temperate and northern species. It is hypothesised that this difference is due to exposure to higher UV doses in clear Antarctic waters. The authors discussed the implications of this finding in the sense that UV radiation can induce photolysis of dissolved organic matter to produce $H_2O_2$ thus exposing aquatic biota to increased oxyradical species (Abele *et al.*, 1998), while also altering animals ability to respond to external stressors. Thus, published studies reveal a wide range of positive effects e.g. increased recruitment and density (Lotze *et al*. 2002,

Molis *et al*., 2003), negative effects e.g. reduced biomass and surface cover

(Wahl *et al.*, 2004, Dobretsov *et al*., 2005), and neutral effects (Hoyer *et al*., 2014) of UV radiation on various aspects of bivalve biology. Species from different geographic regions exhibit varying capabilities to respond to UV, although UV-tolerant species can still be affected by sudden increases in UV exposure (Regoli *et al*., 2000).

**Table 1. Impact of UVR on bivalve species**

| **Study organism** | Treatment | Experiment type [a] | Experiment description | Result | Role of UV in result | Ref. |
|---|---|---|---|---|---|---|
| **Eastern oyster *Crassostrea virginica*** | UV (unspecified) | Unspecified | *C. virginica* transplanted to polluted dock and larvae of these were exposed to UVR | Growth rates reduced in larvae | Not identified | Peachey (2003) |
| **Giant clam: *Tridacna gigas* & dinoflagellates** | UV-A & B | EXCL | Clams with symbiont algae kept in aquaria exposed to natural sunlight. Objective of experiment was to study effect on algae | Results focused primarily on Zooxanthellae population. No biological parameters of clams investigated | Not identified | Buck *et al*., (2002) |
| **Asian clam: *Corbicula fluminea*** | Full spectrum UVR | MAT | Modelling experiment to determine the drivers of larvae dispersal of invasive bivalve | UV radiation is not a main driver of larvae dispersal or mortality. | Not identified | Hoyer *et al.*, (2014) |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Clam spp: *Arca* spp.** | 10% above ambient | SUP | Mylar filters and solar simulators used. Only recorded mortality | No effect observed. | Not identified | Reaka-Kudla *et al.*, (1993) |

| Table 1. Impact of UVR on bivalve species (cont.) | | | | | | |
|---|---|---|---|---|---|---|
| **Blue mussel: *Mytilus edulis* & macrobentic flora/fauna** | 0.258 – 0.384 $W.m^{-1}$ UV-B | SUP | Aquatic settlement panels with UV lamps to investigate impact on community structure, density and biomass | Mussel density positively correlated with UV-B. Effect was not long lived and may have been due to shading algae | Identified<br><br>Species composition & diversity | Mollis *et al*., (2003) |
| **Blue mussel *Mytilus edulis* & algal species** | Solar radiation (UVA, UV-B and PAR)<br><br>0.04 W $m^{-1}$ UV-B | EXCL | Floating raft with wavelength exclusion filters to examine impact of UV on community structure | High UV (August) positively related with mussel recruitment | Identified<br><br>Settlement/ Community structure | Lotze *et al*., (2002) |
| **Blue mussel: *Mytilus edulis* & macrobentic flora/fauna** | Solar radiation (30 $W.m^{-1)}$ UV-B & UV-A | EXCL | Floating raft with wavelength exclusion filters to examine impact of UV on community structure | UV-B reduced diversity and biomass but not as much as UV-A and effects were short-lived | Identified<br><br>Community structure | Wahl *et al.*, (2004) |

| Table 1. Impact of UVR on bivalve species (cont.) | | | | | | |
|---|---|---|---|---|---|---|
| **Green mussel: *Perna viridis* & Horsemussel: *Modiolus comptus*** | Solar radiation UVB & UV-A | EXCL | Floating raft with wavelength exclusion filters to examine impact of UV on community structure | Exposure to UVR reduced the % cover of bivalve species | Not identified | Dobretsov *et al.*, (2005) |
| **Antarctic scallop: *Adamussium colbecki*** | 1.8 W UV-B & 1.0 W UV-A radiation (60 hrs) | SUP | Experiment designed to measure Total Oxyradical Scavenging Capacity (TOSC) in polar and temperate scallop species. | Exposure to UV-A and UV-B significantly reduced antioxidant capabilities in scallops | Identified UV-A + B reduced TOSC by 25 – 35 %. UV-A only was less effective at 15 – 20% | Regoli *et al.*, (2000) |

[a]

Experiment type: SUP = Supplementary i.e. experiment was carried out using supplementary UV emitting bulbs. EXCL = Exclusion i.e. experiment utilised UV blocking filters to compare effect of natural light against specific wavelength excluded light. MAT = Mathematical model utilised to study impact of stressor i.e. no manipulation experiment carried out.

### 2.4.2 Effects of UV on mollusc pathogens

Table 2 displays the studies found which investigated the use of UV to inactivate pathogens commonly found in bivalves. Liltved *et al*. (1995), examined the inactivation of bacterial and viral pathogens, problematic to aquaculture, under UV radiation. It was found that ubiquitous *Vibrio* bacteria, pathogenic to many bivalve species, were sensitive to UV-C irradiation. *V. anguillarium* and *V. salmonicida* required exposure of 1.8 and 1.5 mWs $cm^{-1}$ UV-C, respectively, to achieve 99.9% inactivation. In comparison, the bacterium *Yersinia ruckeri*, primarily a fish pathogen but recently identified in bivalve species (Chistyulin *et al*., 2016), is more resistant and required exposure of 2.7 mWs $cm^{-1}$ UV-C to achieve 99.99% inactivation. Joux *et al.* (1999) investigated the effects of UV-B (0.23 mW $cm^{-1}$) on various bacterial species associated with disease outbreaks in bivalve aquaculture e.g. *Vibrio natriegens* and *Pseudomonas* spp.. These bacteria have been linked to summer mortality syndrome and bacillary necrosis at bivalve production sites (Paillard *et al*., 2004, Garnier *et al.*, 2007, Zannella *et al.*, 2017). It was found that *V. natrigens* was the most sensitive to UV-B, yet also exhibited a 'very effective' UV-A mediated photoreactivation pathway which repaired UV-B induced DNA damage. Zenoff *et al*. (2006), measured the resistance of bacterial species, collected from different altitudes to UV-B radiation (intensity: 0.33 mW $cm^{-1}$, dose: 3.931 kJ $m^{-1}$). Many of the bacteria examined are known to be pathogenic to bivalve species. For example *Actinobacteria,* a group which contains the

'Nocardiosis'-causing *Nocardia,* as well as *Crassostrea,* and *Cytophaga* sp. Which is an aetiological agent of 'Hinge ligament erosion' disease in oysters (Travers *et al.*, 2015, Zannella *et al.*, 2017). Zenoff *et al*. (2006), found that bacterial survival was dependent on strain origin. For example, *Pseudomonas* sp. From the marine environment showed the lowest resistance to the UV-B dose, compared to the high-altitude-originated *Pseudomonas* sp. The authors also measured the recovery of the bacteria studied and found significant variation between recovery strategies independent of habitat of origin. The highest accumulation of CPD photoproducts, and the most efficient recovery, under both light- and dark-treatments, were exhibited in *Acinetobacter johnsonii* and *Cytophaga* sp. Whereas, *Pseudomonas* strains had low survival after UV-B exposure and demonstrated low capacity to recover through photoreactivation.

The infectious pancreatic necrosis virus (IPNV) is pathogenic in fish and has been isolated in bivalves (Mortensen *et al*., 1992, Renault & Novoa, 2004), had a much higher resistance to UV-C than bacteria within the same study (122 mWs $cm^{-1}$ for 99.9% inactivation) (Liltved *et al.*, 1995). UV-C can also inactivate ostreid herpesvirus 1 (OsHV-1) (Schikorski *et al.*, 2011). In the latter study, oyster tissue homogenate containing OsHV-1 DNA was exposed to UV-C (254 nm, 1.08 mW $cm^{1}$) and subsequently injected into healthy oysters, yet no mortality was observed. In comparison, when oysters were injected with OsHV-1 DNA that had not been exposed to UV-C, mortality was recorded. Norovirus, which is harboured in bivalves and is the aetiological agent of many gastroenteritis outbreaks in

humans, can be deactivated by UV-C radiation. Flannery *et al*. (2013), used full spectrum solar radiation to reduce detection of Norovirus (NoV) *via* RT-qPCR by 90% under conditions set to replicate summer (23.5 mW $cm^{1}$, 1.8 kJ $cm^{-1}$ for 21 hrs) and winter (5.6 mW $cm^{-1}$, 0.5 kJ $cm^{-1}$, 89 hrs) in Ireland. Flannery *et al*. (2013), found that natural solar UV, rather than temperature, within the summer conditions, was a greater driver of NoV inactivation and the difference in time required to inactivate 90% of NoV was a direct function of increased irradiance of summer compared to winter. These data suggest that although bacterial, viral, and protozoan pathogens react negatively to UV exposure, the tolerance and recovery capabilities of different pathogens varies between taxa and geographic origin.

**Table 2. Impact of UVR on marine pathogens**

| **Study organism** | Host species | Treatment | Experiment type [a] | Experiment design | Result | Role of UV-B in result | Ref. |
|---|---|---|---|---|---|---|---|
| ***Bacterial pathogens*** | | | | | | | |
| ***Vibrio natriegens Sphingomonas sp. Pseudoalteromonas haloplanktis Deleya aquamarina Pseudomonas stutzeri*** | Fish and shellfish | 950 -1,500 $J.m^{-1}$ UV-B | SUP | Bacteria exposed to UV-B bulbs and left recover in UV-A. Measured resulting CPDs and sensitivity to UV-B | *V. natriegens* is very sensitive to UV-B. Photorepair mechanism evident | Identified<br><br>Inactivation of bacteria. | Joux *et al*. (1999) |
| ***Actinobacteria sp., Pseudomonas sp., Cytophaga sp., Serratia sp.*** | Fish and shellfish | 3.9 kJ $m^{-1}$ UV-B | SUP | Measured bacterial survival, resistance, dimerization and photo-repair rates under UV radiation | Inactivated 63% of bacteria.<br><br>Survival, resistance, CPD formulation and repair occurred in all species but showed diversity | Identified<br><br>Responsible for all results | Zenoff *et al*., (2006) |
| ***Vibrio anguillarum***<br><br>***Vibrio salmonicida***<br><br>***Yersinia ruckeri*** | Fish and shellfish | UV-C (254 nm) | SUP | Measured inactivation of pathogens under short-wave UV | Inactivated 99.9% of bacteria with 1.5 – 2.8 $mWs/cm^{-1}$ | Identified<br>Inactivation of bacteria | Liltved *et al*., (1995) |
| ***Viral pathogens*** | | | | | | | |
| **Norovirus GI, GII F-specific RNA (FRNA) bacteriophage** | Bivalve molluscs (nonpathogenic) and humans | Simulated solar radiation | SUP | Measured inactivation of viral pathogens under full spectrum solar UV | Summer UV conditions reduced detection of NoV and FRNA significantly faster than winter UV conditions. | Identified<br>Inactivation of virus and bacteriophage | Flannery *et al*., (2013) |

**Table 2. Impact of UVR on marine pathogens (cont.)**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **OsHV-1 □Var** | Bivalve molluscs | UV-C 1.08 mW $cm^2$ at 254 nm | SUP | Oyster tissue homogenates containing OsHV-1 □Var were exposed to UV-C and then injected into healthy oyster tissue | UV irradiation of filtered tissue homogenates was successful in removing viral DNA. Healthy oysters injected with UV treated homogenate did not experience mortality. | Identified | Schikorski *et al*., (2011) |
| **Range of viruses & bacteriophages** | Unspecified | Natural solar radiation | EXCL | Investigated rates of decay and infectivity of virus particles under UV | All viruses decayed significantly faster under UV light. | Identified<br><br>Decay of bacteriophages | Noble and Fuhrman (1997) |
| **Viral species LMG1-P4, PWH3a-Pl, LBlVL-Plb** | Unspecified | Natural solar radiation | EXCL | Examined mechanisms and rates of viral decay from solar radiation | All viruses highly sensitive to solar radiation. Decay rates ranged from 2x – 10x higher than dark controls | Identified Decay of viruses | Suttle and Chen, (1992) |
| ***Protozoan parasite*** | | | | | | | |
| ***Cryptosporidium parvum*** | Mammals | 0, 32, and 66 kJ $m^{-1}$ UV-B | SUP & EXCL | Both lab and field experiments using UV-B lamps and UV blocking filters to determine if oocyst infectivity into human cells was affected by UV-B | Artificial dose of 32 and 66 kJ $m^{-1}$ reduced infectivity by 58 and 98%.<br><br>Natural exposure doses or half or full solar intensity reduced infectivity by 67% and >99% | Identified Reduced oocyst infectivity | Connelly *et al.*, (2007) |

1 [a.] Experiment type: SUP = Supplementary i.e. experiment was carried out using supplementary UV emitting bulbs. EXCL = Exclusion i.e. 2 experiment utilised UV blocking filters to compare effect of natural light against specific wavelength excluded light.

### 2.4.3 Effects of UV on Host-Pathogen relationships

Studies investigating the impact of UV radiation on non-bivalve hostpathogen relationships have led to variable conclusions regarding the overall net effect on parasitism (Table 3). Ruelas *et al*. (2009), examined the effect of UV-B on the freshwater molluscan gastropod *Biomphalaria glabrata* and the parasitic relationship with the miracidia of the trematode *Schistosoma mansoni*. It was found that UV-B exposure caused harm to the host, expressed as reduced survival, without affecting the parasite. A variation on this scenario is where exposure to UV radiation negatively affects both parasite and host. An example was observed by Studer *et al.*, (2012) who noted that UVR reduced the survival of parasitic trematode cercariae *Maritrema novaezealandensis* but also increased host susceptibility in the amphipod *Paracalliope novizealandiae*. In this example, the most prominent effect observed was reduced parasite survival, but this may be compensated by reduced host fitness. Overall, the authors note that the net effect of this scenario is likely to be neutral, however, interactive compounding environmental variables would likely affect this interaction *in situ*. Alternatively, UV radiation can reduce parasitism without seemingly affecting the host, as is the case for the

crustacean host *Daphnia dentifera* and its fungal pathogen *Metschnikowia bicuspidata* (Overholt *et al.*, 2012). In the study by Overholt *et al*. (2012), fungal spores were damaged by all wavelengths examined, ranging from shortwave UV-B to longwave UV-A and even PAR. In contrast, the host organism *Daphnia* did not exhibit decreased survival unless an extremely



high UV dose of 32 kJ $m^{-1}$ was administered, compared to the 0.7 kJ $m^{-1}$ required to reduce infectivity of the fungal spores. The authors note that the difference in UV-tolerance between the fungal parasite and the crustacean host is primarily due to the presence of photoenzymatic DNA repair in the host. In other studies, it was found that exposure to both UV radiation and pathogens can synergistically increase negative impacts on hosts. This was observed by Kiesecker & Blaustein (1995), who showed that amphibian embryos experienced higher mortality when simultaneously exposed to UV radiation and a fungal pathogen. Evidently, UVR affects host-pathogen relationships with varied net effects.

Just a single study has included UV radiation as a factor when studying host-pathogen relationships of bivalves. Bettencourt *et al.* (2013), studied the relationship between the palourde clam and *Escherichia. Coli*. The authors found that *E. coli* detection levels were affected by seasonality i.e. detection was lowest during summer months associated with high solar UV radiation, high temperature and low rainfall. However, the data do not conclusively show a specific UV effect.

**Table 3. Impact of UVR on Host – Parasite interactions**

| Study organism | Treatment | Experiment type[a] | Experiment design | Result | Role of UV-B in result | Ref. |
|---|---|---|---|---|---|---|
| ***Aquatic invertebrates*** | | | | | | |
| ***Host:*** **Palourde clam** ***Ruditapes decussatus*** ***Parasite:*** ***Bacteria: Escherichia coli*** | Natural solar radiation | n/a | Clams collected from field throughout year and screened for *E. coli* | Correlation between high temp./ solar radiation with low *E. coli* infection. | Not identified | Bettencourt *et al*., (2013) |
| ***Host:*** **Freshwater snail** ***Biomphalaria glabrata*** ***Parasite:*** **Tremtode:** ***Schistosoma mansoni*** | 500, 625, 750, 825, and 1000 J.m$^{1}$ UV-B | SUP | Examined effect of UV-B on survival of infected snails and susceptibility and resistance of snails to infection | UV-B lowered survival in infected snails. UV-B had no effect on susceptibility or resistance to infection | Identified Survival of infected snails | Ruelas *et al*., (2009) |
| ***Host:*** **Crustacean** ***Daphnia* spp.** | 9.5 – 15 kJ m$^{-1}$ UV-B | SUP | Lab and field experiments to determine susceptibility of host and infectivity of parasite under UVR | UV-B reduced infectivity of parasite but did not | Not identified | : |

| | | | | | | |
|---|---|---|---|---|---|---|
| ***Parasite:*** **Fungal:** ***Metschnikowia bicuspidate*** | | | | alter susceptibility of host to infection. | | Overholt *et al.*, (2012) |

**Table 3. Impact of UVR on Host – Parasite interactions (cont.)**

| | | | | | | |
|---|---|---|---|---|---|---|
| ***Aquatic vertebrates*** | | | | | | |
| ***Host*:**<br>**Wistar rats**<br>***Rattus norvegicus***<br>***Parasite:***<br>**Nematode:** ***Trichinella spiralis*** | 800 mJ<br>(45% UV-B) | SUP | Rats infected with *T. spiralis* exposed to UVR to investigate impact of radiation on infection & immunosuppression | UV-B supresses resistance to parasitic infection. Radiation supresses cellular immune response. | Identified<br>Immunosuppression | Goettsch *et al.*, (1994) |
| ***Host:***<br>**Amphibian species**<br>***Bufo boreas***<br>***Hyla regilla***<br>***Rana cascadae***<br>***Parasite:***<br>**Fungal*:***<br>***Batrachochytrium dendrobatidis*** | 17.9 µW $cm^{-1}$<br>UV-B | EXCL | Investigated combined effect of fungal pathogen &<br>UV-B. Measured frog mortality. | No effect was found from UV-B alone nor synergistic with fungus. | Not identified | Garcia *et al*., (2006) |

| | | | | | | |
|---|---|---|---|---|---|---|
| ***Host:***<br>**Amphibian embryos:**<br>***Bufo boreas, Hyla regilla, Rana cascadae***<br>***Parasite:***<br>**Fungal: *Saprolegnia ferax*** | Natural solar UVB | EXCL | Field experiment to test synergism between exposure to UV-B and fungal pathogen | Results showed synergistic effect of UV-B and pathogen which caused mortality higher than stressor alone | Identified<br>Induced mortality in pathogen & host | Kiesecker and Blaustein, (1995) |
| **Table 3. Impact of UVR on Host – Parasite interactions (cont.)** | | | | | | |
| ***Host:***<br>**Snail: *Zeacumantus subcarinatus***<br>**Amphipod: *Paracalliope novizealandiae***<br>***Parasite:***<br>**Trematode: *Maritrema novaezealandensi*** | PAR + UV-A + UV-B<br>5.84 kJ $m^{1}$ UV-B (>300 nm) | EXCL<br>SUP | Experiment aimed to investigate effect of UVR on trematode transmission through life stages | UVR had negative effect on both survival and infectivity of cercariae and on host susceptibility – Net effect neutral | Not Identified<br>(Results discussed as UVR) | Studer *et al*. 2012 |
| ***Host:***<br>**Fish:**<br>***Gambusia holbrooki***<br>*Pathogen:*<br>**Protozoan: Whitespot**<br>***Ichtyophthirius multifiliis*** | 0.23 W $m^{-1}$ and 0.47 W $m^{-1}$<br>UV-B. UV-A and PAR present | SUP | Experiment designed to test effect of temperature and UV-B on the energy consumption and disease susceptibility | Synergistic effect of high UV-B and high temperature which caused large rise in infection intensity | Identified | Cramp *et al.*, (2014) |

3. Experiment type: SUP = Supplementary i.e. experiment was carried out using supplementary UV emitting bulbs. EXCL = Exclusion i.e. experiment utilised UV blocking filters to compare effect of natural light against specific wavelength excluded light.

## 2.5 Discussion

### 2.5.1 Overview

Empirical research, reviews and meta-analyses have shown that aquatic invertebrates and vertebrates respond to UV with a mixture of negative (decreased survival, impaired metabolism, growth, and reproduction) and neutral or positive (behavioural avoidance and/or defence, and upregulation of molecular defence and/or repair mechanisms) responses. Some evidence exists for UV impacting negatively on bivalve growth (Peachey, 2003), antioxidant capacity (Regoli *et al*., 2000) and surface cover (Wahl *et al*., 2004, Dobretsov *et al*., 2005). In contrast, positive effects of UV exposure included increased density (Mollis *et al*., 2003), recruitment (Lotze *et al*., 2002). No studies could be found which investigated the impact of UV on bivalve health including immune response or pathogen prevalence. There is evidently a noticeable shortage of data on the UV biology of bivalve molluscs, which hinders our comprehension of the effects of such radiation on coastal ecosystems. It is important to understand the effects of environmental conditions on organisms in order to maintain their ecological and economic functions. Furthermore, a better understanding of the impacts of current environmental conditions such as UV radiation is required to better predict animal health in future scenarios of a rapidly changing climate.

Bivalve molluscs face threats from various pathogens and parasites (Walker, 2004, FAO, 2018). Thus, any factor modulating organismal health and immune responses is relevant, especially in the context of the economic and ecological value of bivalve molluscs. Generally, UV

radiation has diverse impacts on host-pathogen relationships. The literature contains case studies of UV-induced increases in infection, generally due to reduced host health or immunosuppression (Ruelas *et al*., 2009), as well as cases of net decreases in parasitism (Overholt *et al*., 2012). Additionally, UVR may have negative effects on both host and parasite, which can render the net effect on parasite transmission to be neutral (Studer *et al*., 2012). While these observations are far from conclusive, they allude to the potential to exploit natural UVR solar disinfection for disease management in the aquaculture industry through optimised management and handling practices. Specific information related to the biology of hosts and parasites is needed to predict outcomes of UVR on systems in question and these should be addressed on a case-by-case basis to avoid making generalisations. Improved understanding of the effects of UV on bivalve – pathogen relationships is required to ascertain whether natural UV can be exploited as a tool for modulating bivalve host-pathogen interactions in shellfish aquaculture.

From the literature reviewed emerges a clear pattern of pathogen responses to UV exposure. A negative 'inactivation' or 'decay' response from bacterial, viral, bacteriophage and protozoan species is widely reported, reducing the overall pathogen presence or infectability. UV exposure has been shown to be detrimental to the survival of common microbes found in bivalves such as *Vibrio* spp., OsHV-1, Norovirus, and *Cryptosporidium*. This finding was expected given the wide-spread commercial application of UV-C in disinfection and sterilisation (Acra *et al*., 1990). However, the

data presented show that UV-sensitivity, for example of *Vibrio natriegens* (Joux *et al.*, 1999), extends into the UV-B wavelengths indicating the potential for natural solar radiation to decrease the risk of water-borne pathogens. It is important to note the variation in dosages used in these experiments ranging from 0.9 kJ $m^{-1}$ UV-B as used to inactivate *Vibrio* spp. (Joux *et al*., 1999) to 3.9 kJ $m^{-1}$ UV-B as used by Zenoff *et al*., (2006) to inactivate *Pseudomonas* spp., up to 66 kJ $m^{-1}$ UV-B as used by Connelly *et al.*, (2007) to reduce infectivity of protozoan oocysts. In 2019, the daily UV-B levels received at the Irish Meteorological Service station in the south west of Ireland ranged from 0.03 kJ $m^{-1}$ to 4.32 kJ $m^{-1}$ (Met Eireann, unpublished data). A better understanding of pathogen inactivation under natural, solar UVR is required in order to apply this process to coastal aquaculture, for example, in determining geographical variability for the purpose of aquaculture site selection.

A common scenario of UV-modulated changes in host-pathogen relationships relates to UV-mediated changes in pathogenicity. However, it is worth noting that an inverse relationship also exists where parasitism modulates UV exposure in bivalves. For example, parasitized cockles *Cerastoderma* spp. And Manila clams *Ruditapes philippinarum* are more often found closer to the sediment surface, where UV levels are higher, compared to healthier counterparts (Thomas & Poulin, 1998, Blanchet *et al.*, 2003, Nam *et al.*, 2018). In another instance, oysters *Crassostrea virginica* and *Ostrea edulis* infected with a surface coating *Vibrio* infection had under-calcified, fragile shells, compared to healthy oysters (Elston *et*

*al*., 1982). Such, parasite-related changes to host biology may leave these animals more vulnerable to a range of environmental factors, and potentially result in exposure to higher UVR doses. These observations show the complexity of the interactions between bivalves, pathogens and UVR.

Full understanding of the effects of UVR on organisms requires knowledge of both dose-response curves and wavelength-response curves (Paul & Gwynn-Jones, 2003). As is the case with much of the photo-ecology literature, a strong bias exists in favour of research based on the use of high UV doses, i.e. research focussed on assessing the impacts of high UV doses associated with stratospheric ozone depletion (Paul & Gwynn-Jones, 2003, Jansen & Bornman, 2012). It is only in some areas of research that the perception of the biological impacts of UV radiation has shifted to the environmental role of low doses of natural UV. These low doses may act as a regulator of specific processes and/or as an environmental cue rather than an 'abiotic stressor' (Jansen & Bornman, 2012). In the field of plant science research, UV is known as a regulator of biochemical pathways by inducing transcriptional responses and signalling pathways (Pontin *et al.*, 2010, Jansen & Bornman, 2012, Krasylenko *et al.*, 2012). However, in zoological studies the potential of UV to act as a intrinsic biochemical modulator and/or immunoregulator has not been fully investigated.

### 2.5.2 Future Outlook

Manipulation of host-pathogen relationships can enhance biosecurity and support sustainable production in the aquaculture sector. UVR is a natural factor that can potentially be exploited to alter host-pathogen relationships. However, the current knowledge base is too narrow to fully comprehend the potential impacts of UVR. More high-quality research is needed with a strong emphasis on accurate reporting of experimental conditions, and especially UV doses and spectra. It is important to use ecologically relevant UV doses i.e. wavelength, intensity, and exposure duration, when investigating potential impacts on host-pathogen relationships *in situ*. Due to bell-shaped dose-response curves, low intensity UVR effects may be the opposite of those observed under high intensity UVR (Aphalo & Albert, 2012). Experiments should also be designed to collate data on sub-lethal effects of UV radiation including immunomodulation and indirect trophic-level effects such as PUFA availability. It would be advisable to include multiple developmental stages in future studies to compare and contrast vulnerabilities and coping mechanisms in different life stages. Furthermore, future studies should look to explore the complex relationship between environment and parasitism in a bi-directional manner. This would allow for better understanding of not only how parasitism is influenced by an animals environment but also how parasitism affects the environment to which an animal is exposed. If it is shown that UV radiation consistently impacts host-pathogen relationships in a predictable manner, then this knowledge can be exploited through optimised management and handling practices. Practical measures to alter UV exposure *in situ* can be achieved by changing the spatial design of bivalve farms, e.g.

by altering the position of stock along the intertidal gradient; by altering structural design of lantern cages, mussel socks or hanging long-lines; or by the use of mesh bags with specific optical properties. In subtidal cultivation, the depth at which animals are kept could be changed in order to optimise UV exposure. Finally, the timing of processes such as laying out new seed may be altered to increase or decrease UV exposure. Thus, were UV to emerge as a significant environmental factor in modulating the host-pathogen relationships of bivalves, practical measures are available to exploit this finding in a commercial setting. With this, efforts can be focused on addressing and advancing biosecurity in shellfish aquaculture and meeting sustainable food security goals for a rapidly growing human population.

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## 2.7 Author contributions

G.F.K: Literature collection, review and analysis, graphic production, Writing – original draft preparation and editing, Methodology, Formal analysis, Validation. S.C: Funding acquisition, Project administration,

1 Resources, Supervision, Conceptualization, Methodology, Writing – 2 reviewing and editing. S.L: Funding acquisition, Project administration,

3 Resources, Supervision, Conceptualization, Methodology, Writing –

4 reviewing and editing. M.J: Funding acquisition, Supervision,

5 Conceptualization, Methodology, Writing – reviewing and editing

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# Chapter 3: Development of a sensitive polymerase chain reaction (PCR) and digoxigenin (DIG)-labelled *in situ* hybridisation (ISH) for the detection of *Vibrio* bacteria in the Pacific oyster *Crassostrea gigas*.

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## 3.1 Abstract

*Vibrio* bacteria are one of the greatest threats to aquaculture with *Vibrio*associated mass mortality events occurring in all life stages worldwide. *Vibrio aestuarianus* is particularly problematic in Pacific oyster *Crassostrea*

*gigas* cultures. A diagnostic method used to detect *V. aestuarianus* is real-time quantitative (q)PCR and a confirmatory method is fluorescent *in situ* hybridisation (FISH). These methods require specialised equipment and incur high costs. The objective of this study was to develop a novel generic PCR and DIG-labelled *in situ* hybridisation (ISH) for the detection and confirmation of *Vibrio*. A PCR that detects all *Vibrio* spp. Is particularly useful for blanket screening of hosts and environmental samples, while ISH facilitates the visual localisation of bacteria. Both diagnostic methods were developed to be used synchronously with other methods that are specific to certain *Vibrio* spp. Primers (VibF3/VibR3) were designed to amplify a 286 bp product from the 16S ribosomal RNA gene common to all *Vibrio* spp. And to form the ISH probe. Confirmatory positive and negative controls were used in both methods. Results were compared with a *V. aestuarianus* specific qPCR protocol. Direct Sangar sequencing of PCR products, Blastn analysis and Clustal Omega alignments were used to confirm *Vibrio* sp. Detection and assess similarity. Pathologies observed in *C. gigas* tissues were similar to those described in other studies. A positive ISH signal was observed in qPCR and PCR positive oysters and no signal was detected in uninfected individuals. These techniques will increase the diagnostic arsenal against this globally significant genus of bacteria.

Keywords: Aquaculture, Bacteria, Diagnostics, *In situ* hybridisation, PCR, *Vibrio*,

## 3.2 Introduction

The global aquaculture sector is challenged with meeting the demands of a rapidly growing population and the need for a more sustainable source of animal protein (FAO, 2018). Emerging diseases are major threats facing the fish and shellfish aquaculture industry and climate change is predicted to increase the severity of this issue (Marcogliese, 2001, Callaway *et al.*, 2012).

Marine mollusc aquaculture is valued at $20.5 billion USD and the bivalve aquaculture sector supports around 200,000 livelihoods globally (Carnegie *et al*., 2016, Soon and Ransangan, 2019). Infectious diseases and mass mortality events are reported worldwide causing significant economic losses and bringing about the collapse of local industries (Carneige *et al*., 2016, Soon and Ransangan, 2019). Bacterial pathogens within the genus *Vibrio* are of major concern to humans (Austin, 2010, Osunla and Okoh, 2017), fish, and aquatic invertebrates (Austin and Zhang, 2006). *Vibrio* bacteria are ubiquitous in the aquatic environment and pose a threat to both wild populations and farmed animals (Hunt *et al*., 2008, Le Roux, 2016). *Vibrio* bacteria have been associated with significant losses in fish and shellfish aquaculture industry due to high rates of fish and mollusc mortality at all life stages in Europe, America, and Asia (Dubert *et al*., 2017, Garnier *et al*., 2007, Elston *et al*., 2008). It is predicted that the emergence of *Vibrio* related disease outbreaks is likely to increase in the coming years ass climate change increases the geographical and host range of pathogens (Beaz-Hidalgo *et al*., 2010, Vezzulli *et al*., 2016), while also damaging host immune functioning (Martin *et al*., 2010)

Vibriosis, a term which replaced the initially proposed denomination 'bacillary necrosis' (Tubiash *et al*., 1970) is used to refer to disease caused by pathological infection of host species by *Vibrio* bacteria (Elston *et al*., 2008). Vibriosis is characterised by bacterial colonization of the gills, mantle and other internal tissues, visceral atrophy, velum disruption and abnormal swimming in bivalve larvae (Romalde and Barja, 2010). Vibriosis particularly affects bivalve hatcheries and nurseries, where it can cause high mortality in both early and commercial-ready life stages (Dubert *et al*., 2017). *Vibrio aestuarianus* is a pathogenic species of bacteria which has been associated with bivalve mass mortality events around Europe (Garnier *et al*., 2007, Barbosa Solomieu *et al*., 2015, EFSA, 2015). Detection rates of *V. aestuarianus* alongside increased bivalve mortality have been increasing since 2011 in France, Scotland, and Ireland (McCleary and Henshilwood, 2015, EFSA 2015, Travers *et al*., 2017). Mortality associated with *V. aestuarianus* typically occurs in summer as proliferation and spread of the bacteria increases in warmer seawater ranging from 9 – 13 $^{o}$C in colder climates and 19 – 20 $^{o}$C in warmer areas (EFSA, 2015, O' Toole *et al*., 2017, Lupo *et al*., 2019). Histopathology associated with *V. aestuarianus* infection in Pacific oysters includes symptoms such as haemocytic accumulation and infiltration of sinuses (McCleary & Henshilwood, 2015, Mandas *et al*., 2020). Although the exact cause of mortality is poorly studied (Lupo *et al*, 2019), *V. aestuarianus* is known to colonize haemolymphs in early stages of infection, infect the gill and mantle tissues, and lead to septicaemia (Parizadeh *et al*., 2018, Lupo *et al*., 2019, Mandas *et al*., 2020).

The development of rapid, effective, and affordable techniques to identify and localise pathogens is important for early and accurate diagnosis and for a better understanding of disease aetiology and impacts (EFSA, 2010, 2015). Molecular tools are increasingly important in pathogen diagnoses as they have numerous advantages over culture methods e.g., higher specificity, utility, and reproducibility (Saulnier *et al*., 2017). Polymerase chain reaction (PCR) allows for rapid and cost-effective amplification of nucleic acids, which has numerous applications including pathogen detection, sequencing, and genotyping. Quantitative PCR (qPCR) is an advanced method of PCR which utilises a fluorescent probe to quantify amplification of DNA instantaneously (Yang and Rothman, 2004, Lynch *et al*., 2013, Erlich, 2015). Pathogens such as *V. aestuarianus*, *V. splendidus*, and *V. harveyi* are not listed as pathogens by OIE or EU (Directive 2006/88/EC), and no detection nor diagnostic methods are universally recommended. Commonly applied diagnostic methods include real time qPCR (Saulnier *et al.*, 2009, McCleary and Henshilwood, 2015, Saulnier *et al*., 2017), multiplex PCR (Kim *et al*., 2015), alternatively bacterial cells may also be cultured on agar plates (Mandas *et al*., 2020) or counted individually by flow cytometry (Lupo *et al*., 2019). The real-time qPCR, used in this study as a known standard, was developed with a highly specific hydrolysis probe using minor groove binder (MGB) chemistry (McCleary & Henshilwood, 2015). This form of PCR results in highly specific PCR amplification and limits non-specific results (Andersen *et al*., 2006).

*In situ hybridisation* (ISH) is a rapid and cost-effective nucleic acid hybridisation technique used to identify and localise specific gene segments within morphologically preserved tissues and cells using standard light microscopy (Jin and Lloyd, 1997, Lynch *et al*., 2013). With regards to pathogen detection, advantages of ISH include localising the target sequence within the tissue sample and obtaining a better image of the infection spread and severity, along with pathological symptoms of the infection. Digoxigenin (DIG) is a steroid-compound used for labelling probes, which can then be used for fluorescent, colorimetric, or luminescent detection. DIG-labelled probes offer many benefits over radioactive-labelled probes such as increased safety, high versatility, and high specificity (Jin & Lloyd, 1997). Despite *V. aestuarianus*, *V. splendidus*, and *V. harveyi* being significant pathogens of valuable commercial shellfish species, no DIG-labelled *in situ hybridisation* (ISH) method has been developed for the detection of *Vibrio* species.

Although protocols are available for fluorescent *in situ* hybridisation (FISH) (e.g. (Zhang *et al*., 2015) and for real-time qPCR (e.g. (Saulnier *et al*., 2017), these methods often incur high cost, use of dangerous materials and specialised equipment. The standard PCR and ISH described here are lowcost, rapid, and effective methods for the screening and localisation of *Vibrio* bacteria in aquatic organisms.

The aims of this study were to a) design a rapid, inexpensive generic PCR test for the screening of *Vibrio spp.*, b) use the novel PCR primers to develop a DIG-labelled ISH for *Vibrio* spp. in *C. gigas* to facilitate the detection of these pathogens in specific tissue localisations. The development of an efficient and

cost-effective PCR and a safe, reliable DIG-labelled ISH increases accessibility of these diagnostic tools which are vital for reliable pathogen monitoring and assessing pathogen development and behaviour within the host. These additional diagnostic tools will facilitate more informed decision making in relation to management of these bacteria in the marine environment. These methods will be valuable for aquaculture production sites and hatcheries where blanket PCR pathogen screening is useful prior to targeted, specific, and more expensive qPCR screening.

## 3.3 Methodology

### 3.3.1 Oyster sampling and sample site

DNA and tissue sections of commercial and control oysters were subjected to a four-step comparison method to validate the newly described methods (PCR and ISH) against known confirmation methods (qPCR and genome sequencing) (Fig. 1). Pacific oysters *Crassostrea gigas* (N = 125, mean weight: 3.5 g, range: 0.5 g – 10.0 g) were collected from Dungarvan bay, Co. Waterford, Ireland (52.0936 °N, 7.6204 °W) where they had been growing for a minimum of 3 months. This 'Commercial cohort' of oysters were collected over summer months in recent years (2017 – 2018). A separate 'Negative control' cohort (n = 12) comprised archived samples collected during the winter months in previous years (1999 – 2000) and was unlikely to have been exposed to *Vibrio* spp. Negative control oysters originate from a nursery seed supplier free from *Vibrio* and herpesvirus pathogens. All commercial oysters originate from a commercial oyster production site which

has endured mass mortality events linked to both Ostreid herpesvirus – 1 (OsHV-1 µVar) and *Vibrio aestuarianus* since 2003 (Lynch *et al*., 2012, Morrissey *et al*., 2015, Bookelaar, 2018). *Vibrio splendidus* has also been detected at this site, although not yet linked with mortalities (Lynch *et al*., 2021, Notaro *et al*., 2021). With fresh samples, a 5 $mm^2$ piece of gill and internal tissues was removed from each oyster and stored at -20 ºC until DNA extraction *via* the Chelex-100 methodology (Sigma) (Walsh, *et al*., 1991, Lynch *et al*., 2013). Archieved samples underwent deparrafinization as described in Shi *et al*., (2002) before undergoing identical storage and DNA extraction processes as with the fresh samples. Diagonal cross sections (1 $cm^2$) of each oyster were fixed and processed for histological/ISH examination as per guidelines described in Kim *et al*., (2006).

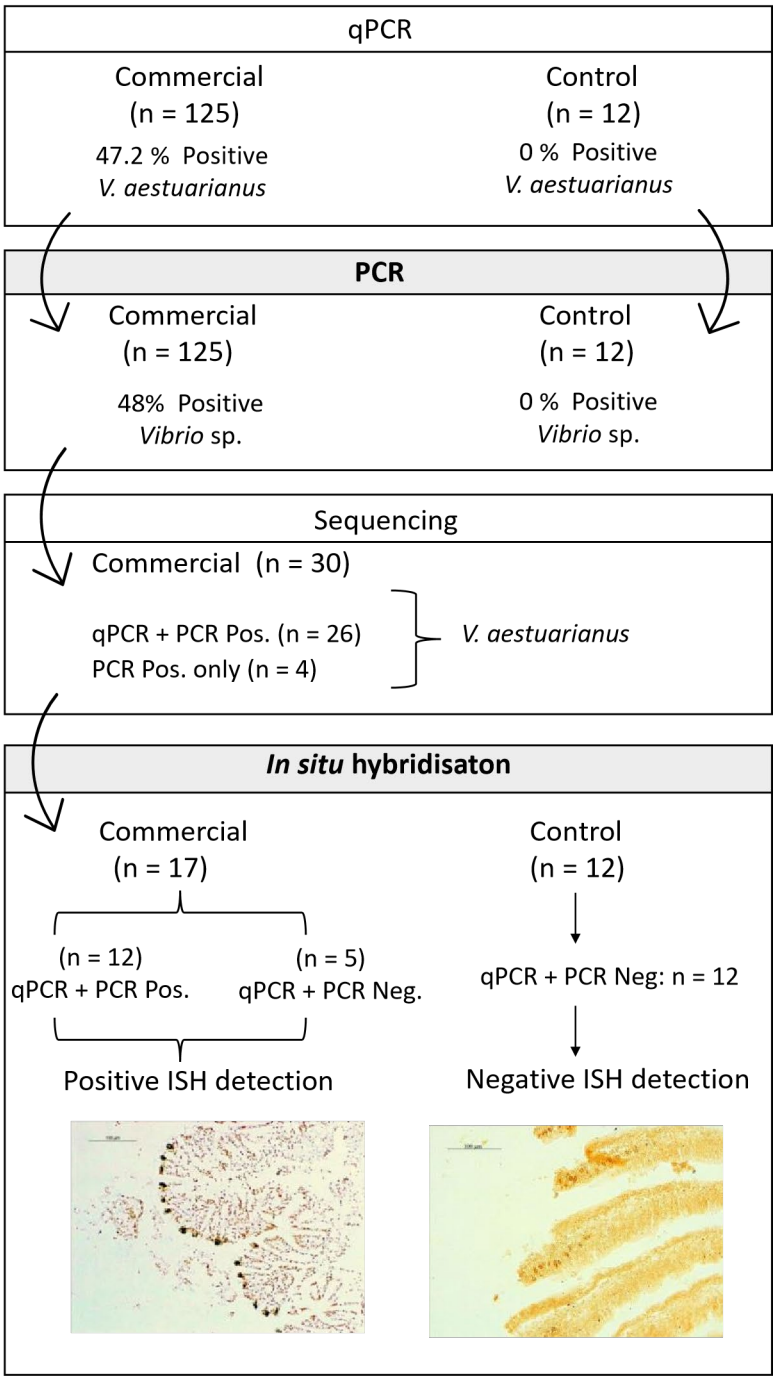



Figure 1. Schematic description outlining the samples screened and the methodology (qPCR and genome sequencing) used and compared to validate the new diagnostic tools (PCR and in situ hybridisation). Pos. = Positive for

infection, Neg. = Negative/Uninfected.

### 3.3.2 Molecular assays

#### *3.3.2.1 DNA extraction*

DNA was extracted from oyster tissue samples using the 10% chelex method carried out at 99 ºC for 1 hr 10 mins (Walsh *et al*., 1991, Lynch *et al*., 2012). DNA samples were stored at -20 ºC. To ensure that the quality and quantity of extracted DNA was optimal for qPCR and novel PCR, a NanoDrop 100 spectrophotometer was used to check the OD 260/280 and concentration of DNA in samples, as per the manufacturers protocol (Wood, 1983).

#### *3.3.2.2 Quantitative polymerase chain reaction (qPCR)*

Real-time quantitative PCR (qPCR) was carried out to determine if oysters were infected or not and to quantify the bacterial load of *V. aestuarianus* in oyster tissue (McCleary & Hensilwood, 2015). The primers used in this qPCR mix (*dnaJ* f420, *dnaJ* R456) are specific to *V. aestuarianus* and the Taqman probe using MGB chemistry ensures high specificity for the desired target (McCleary & Hensilwood, 2015). Oysters were classified into four infection intensity groups based on their Ct value with a Ct of >37 being classified as 'Uninfected' (as per McCleary & Hensilwood, 2015), Ct 35 – 37 as 'Low infection', Ct 30 – 35 as 'Medium infection' and Ct <30 as 'High infected'. Negative controls contained distilled, autoclaved $H_2O$ instead of DNA and positive controls contained 5 □l of purified *Vibrio aestuarianus* DNA (Marine Institute, Ireland).

### 3.3.3 Novel polymerase chain reaction (PCR)

#### *3.3.3.1 Primer design*

Three primer pairs (Table 1, Figure 1) were designed using PrimerBlast tool (NCBI, 2019) optimising important variables such as primer length, annealing and melting temperatures, G:C content, and primer concentration to increase the specificity and robustness (Abd-Elsalam, 2003, Lynch *et al*., 2013). Primers were selected to amplify a sequence from the 16S ribosomal RNA gene region common to all *Vibrio* spp. Optimal primers were chosen to be 20 bp in length, with a melting temperature of 58 ºC to 62 ºC, and with a Guanine:Cytosine (G:C) content of 55 – 60%. Primers were checked for specificity using Primer BLAST (NCBI, 2019).

**Table 1. Primers selected for conventional PCR of *Vibrio aestuarianus***

| Name | Sequence | Length | Start | Stop | Tm | G:C (%) | S.C | P.S. (bp) |
|---|---|---|---|---|---|---|---|---|
| VibF1 | TCAGTTGTGAGGAAGGGGGT | 20 | 415 | 434 | 60.40 | 55.00 | 3.00 | 319 |
| VibR1 | GTGTCAGTGTCTGTCCAGGG | 20 | 733 | 714 | 59.97 | 60.00 | 4.00 | 319 |
| VibF2 | CATGCAAGTCGAGCGGAAAC | 20 | 20 | 39 | 60.18 | 55.00 | 4.00 | 419 |
| VibR2 | CGTTACCCCCTTCCTCACAA | 20 | 438 | 419 | 59.31 | 55.00 | 3.00 | 419 |
| VibF3 | CAACAGAAGAAGCACCGGCT | 20 | 465 | 484 | 60.89 | 55.00 | 4.00 | 286 |
| VibR3 | CACGCTTTCGCATCTGAGTG | 20 | 750 | 731 | 59.91 | 55.00 | 3.00 | 286 |

Tm – Primer melting temperature
S.C. – Self complementarity
P.S – Product size

#### *3.3.3.2 Novel PCR reaction mix and thermocycling conditions.*

Optimal design of the PCR followed guidelines as set out by Apte and Daniel, (2009), Lorenz, (2012) and Lynch *et al*., (2013). The temperatures and

durations of the denaturation, annealing and elongation steps are optimised according to G:C content and length of the primers used (Abd-Elsalem, 2003, Lorenz, 2012).

PCR reaction mix and thermocycling conditions were designed to amplify *Vibrio* spp. genomic DNA and test the suitability of the primers for both PCR and ISH probe synthesis. The PCR reaction mix contained 2.5 µl of genomic DNA, 0.25 µl each of forward and reverse primers (100 pmol/ml), 0.1 µl of GoTaq, 0.5 µl $MgCl_2$ (25 mM), 1.5 µl of DMSO, 5 µl of 5x Green buffer, 5 µl of DNTPs (0.2 mM), and 12.9 µl of distilled, autoclaved $H_2O$ per each individual oyster being screened. Negative controls were used which contained distilled, autoclaved $H_2O$ instead of DNA and positive controls contained 2.5 µl of purified *Vibrio aestuarianus* genomic DNA (Marine Institute, Ireland). The thermocycling conditions followed methods in Lynch *et al*. (2012). Expected product sizes were 319 bp, 419 bp, and 286 bp for VibF1/VibR1, VibF2/VibR2, and VibF3/VibR3 respectively (Table 1) (Fig. 2). To validate the standard PCR, DNA samples (n = 125) were initially screened using a qPCR specific to *V. aestuarianus* (McClearly & Henshilwood, 2015) and results were compared to those from the novel VibF3/VibR3 PCR. The cycle threshold cut-off point (Ct: 37) determines that values greater than 37 are negative for *V. aestuarianus* and values less than 37 are positive for *V. aestuarianus* (McClearly & Henshilwood, 2015). qPCR positive samples were sequenced and the % match was confirmed to be a known *V. aestuarianus* strain (Genbank: AF172840).

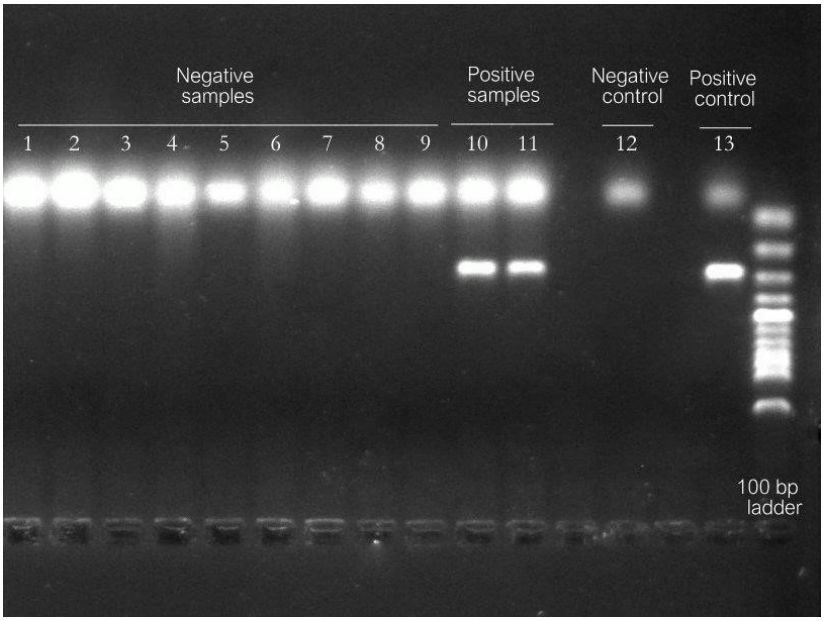



Figure 2. Agarose gel showing PCR product at 286 bp using F3/R3 primers. Columns L:R show *Vibrio* negative samples (no. 1 - 9), *Vibrio* positive samples (no. 10, 11), a negative control ($dH_2O$, n. 12), and a positive control (*V. aestuarianus* DNA, no. 13).

### 3.3.4 DNA sequencing

Direct Sangar sequencing was carried out on PCR products (n = 30) amplified from the VibF3/VibR3 primers to confirm the detection of *Vibrio* sp. PCR products were excised from the agarose gel and then isolated and purified using a gel extraction kit (Qiagen QiaQuick gel extraction kit) prior to sequencing (Lynch *et al*., 2013). Both forward and reverse strands of purified DNA were sequenced commercially (Eurofins Genomics, Ireland). Each sequence was matched against a nucleotide database (https://blast.ncbi.nlm.nih.gov) to confirm that the sequences were a *Vibrio* sp. To further confirm the identity of certain sequences that matched with *Vibrio* sp. in GenBank, those sequences were aligned with confirmed *V.*

*aestuarianus* sequences using Clustal Omega alignment software (EMBLEBI accessed at https://www.ebi.ac.uk).

### 3.3.5 Novel DIG-labelled in situ hybridisation (ISH)

#### *3.3.5.1 Tissue preparation*

Oyster tissue samples were fixed using Davidsons's fixative for 24 - 48 hours and stored in 70% ethanol. Samples were dehydrated and embedded in paraplast (Sigma), cut using a microtome at 7 µm thickness and placed on clear glass slides for light microscopy or on Silane-prep slides (Sigma) for ISH as per guidelines described in Kim *et al*. (2006), and Lynch *et al*. (2013).

#### *3.3.5.2 Probe design*

Once amplification in the PCR (n = 20 oysters) was successful and the specificity of the primers and PCR was confirmed by sequencing (n = 10 samples, n = 6 sequences generated), the remaining PCR products were used as a template to create a digoxygenin-labelled probe for ISH. This was done by repeating the conditions of the novel PCR mix but with the inclusion of the PCR-DIG labelling mix (Roche PCR DIG Probe Synthesis Kit) instead of the dNTPs. Half of the product (12 µl) was visualised by agarose gel electrophoresis for 60 mins at 110 V to ensure successful amplification and binding of the probe to the target DNA i.e. *Vibrio*. The remaining half of the product was used as the DIG-labelled probe in the ISH reaction.

#### *3.3.5.3 DIG-labelled in situ hybridisation*

ISH was performed on samples which had been screened for *V. aestuarianus* with qPCR and then classified as 'Low infected', 'Medium infected', 'High infected' and 'Uninfected' individuals based on their Ct values with a Ct > 37 reflecting an uninfected sample as per the qPCR reference publication

(McClearly & Henshilwood, 2015). The ISH was modified for optimisation following supplier guidelines (Abcam, 2018) and previous work in Russell & Sambrook (2001) and Lynch *et al*. (2010). Solutions and buffers were made up according to Russell & Sambrook (2001) and Abcam, (2018). Silane-prep slides with sections of *C. gigas* tissue adhered to them, underwent deparaffinisation, denaturation and hybridisation with the diluted probe as per the protocol in Abcam (2018). Staining, with nitroblue tetrazolium and 5bromo-4-chloro-3-indolyl phosphate (NBT/BCIP), and counter-staining, with Bismarck Brown Y, were carried out according to Lynch *et al*. (2010). Slides were then mounted with Eukitt mounting medium and, once fully dry, were visualised using a Leica suite microscope and images were captured with NIS-Elements imaging software (Nikon).

## 3.4 Results

### 3.4.1 PCR validation

Of the 125 commercial oysters screened using qPCR, 59 (47.2%) were deemed positive for *V. aestuarianus.* Using the novel VibF3/VibR3 PCR primer pair 60 (48%) of the same 125 samples were deemed positive. 119 out of the 125 samples (95.2%) gave PCR results consistent with those from qPCR, leaving 6 ‘mismatch’ samples, which did not show PCR results consistent with those obtained using qPCR. In two of the six ‘mismatch’ samples, PCR did not detect *Vibrio* DNA where qPCR did detect *V. aestuarianus*. The Ct of these samples were between 34 and 35 indicating a low infection prevalence. In the remaining four of the six mismatch samples, the novel PCR did detect *Vibrio* DNA while the qPCR result was negative for

*V. aestuarianus*. Three of these four samples gave Ct values only slightly above the "uninfected" cut-off point of 37 (37.3 – 37.7) with the other sample being deemed "Undetectable". The positive PCR products from the 6 mismatched samples were sequenced and confirmed to be *V. aestuarianus*.

**Table 2. Overview of PCR and qPCR screening results. [a]**

| Diagnostic result | No. of individuals | % of total |
|---|---|---|
| **qPCR +** | 59/125 | 47.2 |
| **qPCR -** | 66/125 | 52.8 |
| **PCR +** | 60/125 | 48 |
| **PCR -** | 65/125 | 52 |

[a.] qPCR results obtained by carrying out method described in McCleary & Henshilwood (2015). PCR results obtained through novel VibF3/VibR3 primers. + denotes a positive detection of *V. aestuarianus* (qPCR) or *Vibrio* sp. (PCR) while – denotes a negative result.

### 3.4.2 *In situ* hybridisation validation

To evaluate the ISH methodology, *C. gigas* tissue slides were prepared from a subsample of both positively and negatively infected individuals, as determined with qPCR, PCR, and genome sequencing (Commercial cohort: n = 12 positive infection, n = 5 negative infection), and negative control winter archive samples (Negative control group: n = 12) (Table 3). Samples were defined as infected or uninfected based on qPCR, PCR, and genome sequencing results. Each of the 12 (100%) PCR and qPCR positive infected

samples showed positive binding of the DIG-labelled ISH probe to nucleic acids within the tissue sections, particularly around the gills and mantle (Fig. 3). In the 5 samples sourced from the commercial cohort, which tested negative *via* PCR and "borderline" negative *via* qPCR, all 5 showed some binding to nucleic acids in similar tissue sections. "Borderline" negative samples describe the marginal Ct values obtained by qPCR which were within 0.5 of the threshold for the "uninfected" cut-off point of 37 +/- 0.5 None of the 12 negative control samples sourced from the winter archive showed positive binding to nucleic acids (Fig. 4).

**Table 3. *In situ* hybridisation results compared to PCR AND qPCR positive or negative samples.**

| | **Total N** | **ISH Positive** | **ISH Negative** |
|---|---|---|---|
| **Commercial cohort (qPCR & PCR Pos.)** | 12 | 12 | 0 |
| **Commercial cohort (qPCR & PCR Neg.)** | 5 | 5 | 0 |
| **Negative control (PCR & qPCR Neg.)** | 12 | 0 | 12 |

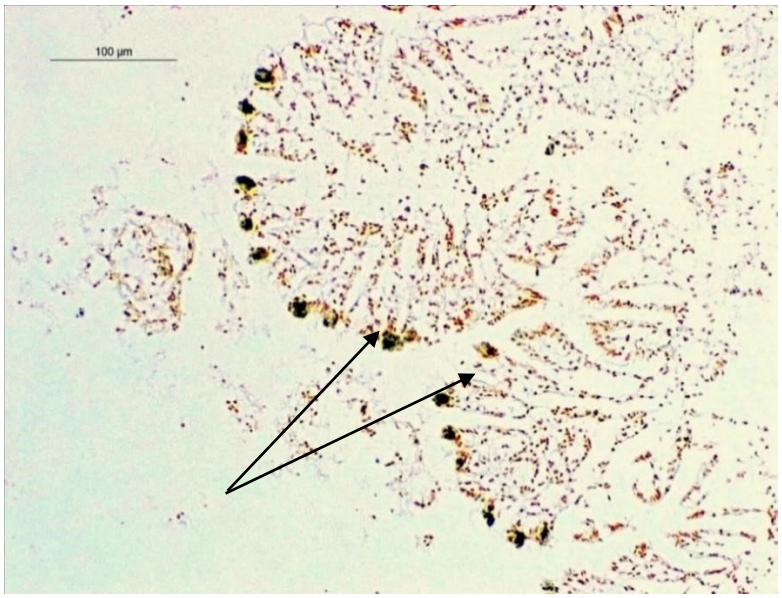



Figure 3. ISH image of Pacific oyster *C. gigas* gill tissue showing high infection with *V. aestuarianus*. Arrows point to clusters of cells which reacted to ISH. qPCR Ct value: 28, PCR result: Positive, sequenced to match: *V. aestuarianus* (40x magnification).




Figure 4. ISH image of Pacific oyster *C. gigas* gill tissue showing no infection from *Vibrio* DNA. qPCR Ct value: "Undetectable", PCR result:

9 Negative. (40x magnification).

### 3.4.3 Microscopy

Inspection of tissue sections of *V. aestuarianus* infected (n = 10) and uninfected (n = 5) (qPCR and PCR verified) oysters *via* light microscopy showed the presence of dispersed bacteria-like cells within the connective tissue and around digestive organs (Fig. 5). Pathology of infected individuals included diapedesis - the leaking of haemocytes into sinuses, typical of inflammation caused by vibriosis (Fig. 5, 6) (Parizadeh *et al*., 2018). Haemocytes were also seen to accumulate in the connective tissue of infected individuals (Fig. 6), symptomatic of bacterial infections (Mandas *et al*., 2020). Gill tissue appeared to have reduced cilia in infected individuals (Fig. 7B) compared to healthy ciliated gills in uninfected individuals (Fig. 7A). There was heavy degradation in the lumen of digestive diverticula of highly infected individuals (Fig. 8B) compared to uninfected individuals (Fig. 8A).

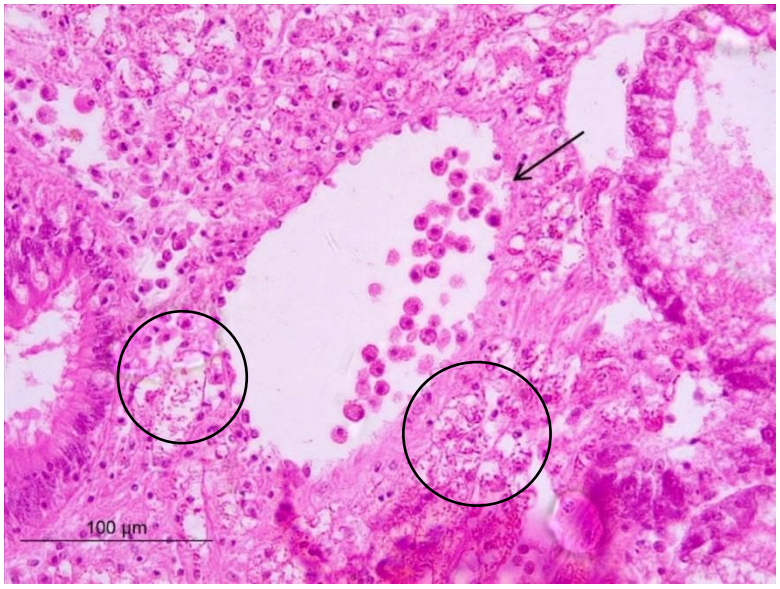



Figure 5. High infected tissue showing diapedesis or blood infiltration into the sinus (arrow) and possible bacterial cells dispersed throughout (circles).

Image taken from commercial oyster shown to be qPCR and PCR positive (Ct: 28.9).

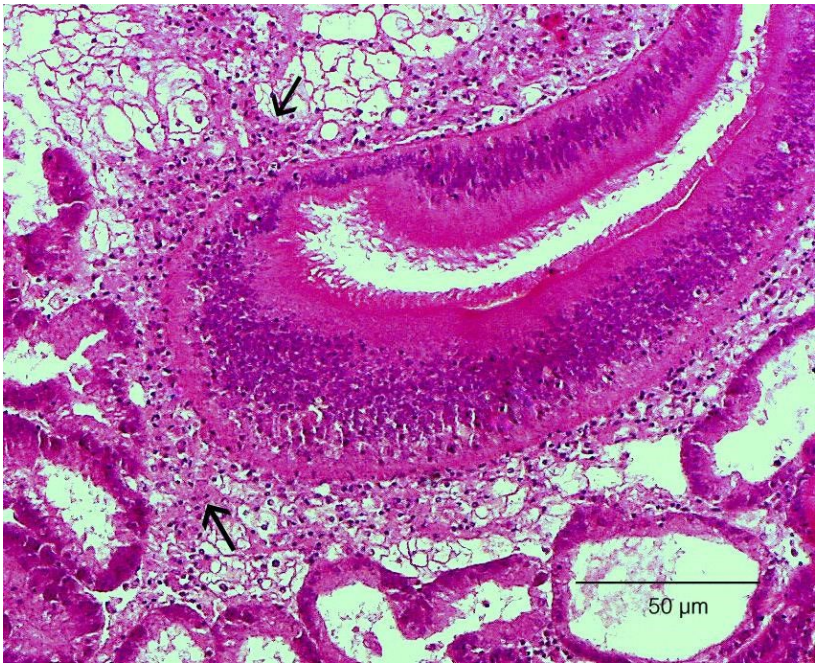



Figure 6. High infected tissue showing haemocyte accumulation in connective tissue (arrows). Image taken from a commercial oyster shown to be qPCR and PCR positive (Ct: 18.4).

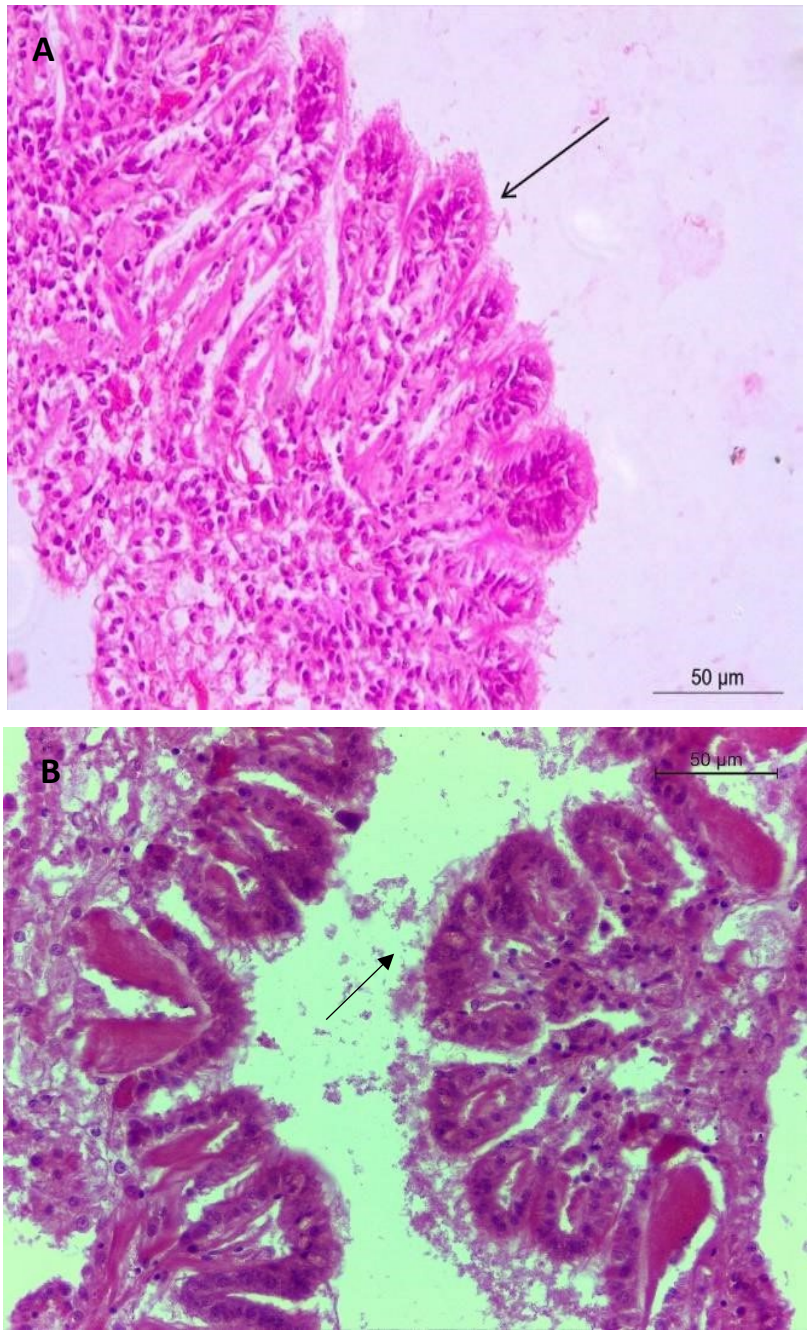



Figure 7. A: Uninfected gill tissue showing healthy cilia (arrow) on gills and no visible bacterial cells from commercial oyster deemed to be PCR and qPCR negative (Ct: 37.5). B: Gill tissue of a heavily infected commercial oyster showed sloughing of cilia (arrow), PCR, and qPCR positive (Ct: 22.2).

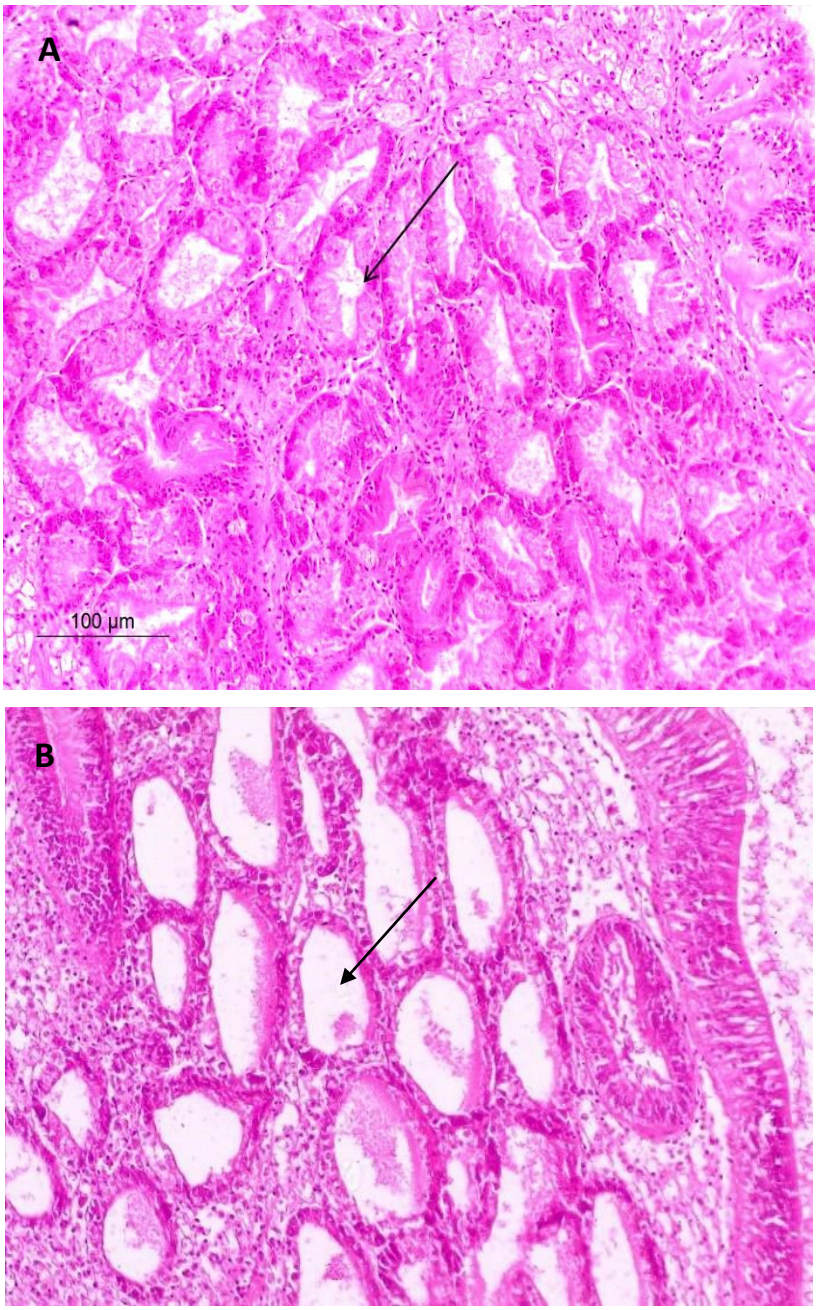



Figure 8. A: Digestive diverticula showing healthy lumen from Uninfected commercial oyster (arrow), deemed negative by PCR and qPCR (Ct: 37.5). B: High infected Digestive diverticula showing poor lumen lining characteristic of infection from *Vibrio* spp (arrow). (similar to observed in Garnier *et al*. (2008)) from a commercial oyster deemed to be PCR and qPCR

positive (Ct: 19.6).

## 3.5 Discussion

In this study, a novel PCR and DIG-labelled ISH for the detection of *Vibrio* spp. including *Vibrio aestuarianus* was developed and validated. Although *V. aestuarianus* was the only *Vibrio* species identified in the cohort of animals used in this study, this PCR has been used in previous studies and successfully detected *V. splendidus* in *C. gigas* samples (Lynch *et al*., 2021, Notaro *et al*., 2021,) as well as in microalgal cultures and seawater samples (Notaro *et al*., 2021). This novel PCR method, and its diagnostic ability was a strong match (>95% consistency) with the standardised qPCR diagnostic test for *V. aestuarianus* as developed by the National Reference Laboratory in Ireland (McClearly & Henshilwood, 2015). In a cohort of 125 samples of DNA extracted from oyster tissue, qPCR detected a *V. aestuarianus* infection prevalence almost identical to the generic PCR (47.2% vs 48% respectively).

The majority of PCR positive samples (70%) which were sequenced were confirmed to be *V. aestuarianus* with a high degree (>95%) of sequence matching. The remaining PCR samples (30%), initially sequenced to *Vibrio* spp. were later confirmed to also be *V. aestuarianus* by Clustal Omega alignment with confirmed *V. aestuarianus* sequences. The previously undescribed method for *in situ* hybridisation of *Vibrio* bacteria including *V. aestuarianus* successfully localised *Vibrio* nucleic acids in the tissue sections of *C. gigas*. All samples which displayed positive infection through PCR or qPCR, were confirmed positive *via* ISH. Positive ISH detections were also observed in 5 samples previously deemed ‘uninfected’ *via* qPCR and PCR.

These samples comprised animals from the same cohort as the infected animals. This suggests that the ISH is capable of detecting very low levels of bacteria and latent infections which were otherwise not detected by PCR and qPCR. ISH has a particular advantage over PCR and qPCR in this respect, as the latter methods are prone to sampling bias and false negatives by way of small samples of DNA taken from a specific tissue sample, allowing pathogen DNA to go undetected elsewhere in the host (Bacich *et al*., 2011, Lynch *et al*., 2013). Alternatively, ISH provides an overview of the entire cross-section of the host animal, allowing the examination of additional internal tissues. It is also very useful for the detection of latent (early) or light intensities of infection. The twelve negative control samples did not show any positive ISH signal. These samples came from the archived (1999 – 2000) winter cohort of *C. gigas*. *V. aestuarianus* is a relatively new pathogen to the shellfish aquaculture industry, being associated with mass mortality events of *C. gigas* in 2001 in France when temperatures reached 19 ºC (Lacoste *et al*., 2001, Alfjorden *et al*., 2017) and in Ireland and Scotland during the summer months of 2011 (O'Toole, 2017, Lupo *et al*., 2019). It seems then that samples deemed negative for *Vibrio* when collected during the summer months, may indeed have been harbouring low levels or latent *Vibrio* infections, this would be supported by the qPCR Ct values which were slightly above the "uninfected" cut-off point. However, the archived winter samples allowed us to validate the ISH methodology and confirm that in samples where no *Vibrio* is present, no ISH staining occurs.

A limitation of PCR is that it cannot confirm that the pathogen is in a viable state, as it might be detecting fragments of the DNA, nor can it provide any information about the spread or behaviour of the infection within the host.

For this reason, molecular and visual tools such as ISH are important to confirm the pathogen infection and supply further information about where and how the pathogen infects the host (Aranguren and Figueras, 2016). ISH can be a vital tool to advance disease characteristics as it allows for better understanding of routes of infections and internal pathogen migration (Le Roux *et al*., 2016). The newly developed ISH method in this study proved successful in binding to *Vibrio* nucleic acids allowing for their localisation within oyster tissues. These novel tools will support the accurate testing for the presence or absence of *Vibrio aestuarianus* and other *Vibrio* spp. and will add to the diagnostic arsenal for *Vibrio* spp. to better understand *Vibrio* hostpathogen interactions, and to tackle future disease outbreaks. This research should prove useful to researchers and industry partners who aim to blanket screen and identify temporal or spatial variances in *Vibrio* levels and localise *Vibrio* within host tissues without using highly specialised or expensive equipment.

## 3.6 Acknowledgements

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## 3.7 Author contributions

G.F.K: Data curation, visualisation, and analysis, graphic production, Writing – original draft preparation, reviewing, and editing, Methodology, Formal analysis, Validation. S.C: Funding acquisition, Project administration, Resources, Supervision, Conceptualization, Methodology, Writing – reviewing and editing. M.J: Funding acquisition, Supervision, Conceptualization, Methodology, Writing – reviewing and editing. S.L: Funding acquisition, Project administration, Resources, Supervision, Conceptualization, Methodology, Writing – reviewing and editing.

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# Chapter 4: The impact of UV-B radiation on Pacific oyster *Crassostrea gigas* health and pathogen *Vibrio aestuarianus* development.

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## 4.1 Abstract

UV-B (□280 – 315 nm) radiation is an important aquatic ecosystem regulator which influences animal motility and orientation, immune health, and mating behaviour. However, UV-B can also induce adverse genotoxic effects on microbial, plant and animal life, including on surface water pathogens such as *Vibrio* species which can be pathogenic to humans and aquatic animals e.g., *V. cholerae, V. splendidus*, and *V. aestuarianus*. Thus, UV-effects on organisms are multifaceted, and can be positive or negative. Pacific oysters *Crassostrea gigas* are typically cultured in the intertidal zone and experience extreme fluctuations in environmental conditions including increased variations in exposure to UV radiation during the diurnal tidal flow. *C. gigas* stocks experience mortality events during the summer months, and these are typically associated with environmental stressors such as elevated sea and air temperature, high salinity, low nutrient availability and pathogens such as protozoa, viruses and bacteria including from the *Vibrio* genus such as *V. aestuarianus* and *V. splendidus*. The role of UV-B in such mortality events or in contributing to underlying physiological pathologies is unknown. To assess the impact of UV-B exposure this study used lab-based experiments whereby *C. gigas* seed were exposed to i) a short, high intensity UV-B dose while submerged in seawater or ii) a longer, low intensity UV-B exposure while the oysters were aerially exposed. Impacts on oyster survival and development of *Vibrio aestuarianus* and related and unrelated pathologies were measured. *V.*

*aestuarianus* infected oysters exhibited pathologies commonly associated with vibriosis such as diapedesis, haemocyte accumulation, tissue degradation and digestive gland atrophy. UV-B exposure did not affect oyster tissue structure and morphology. Yet, results from both low and high intensity exposure trials showed that UV-B exposure increased the rate of mortality in oyster seed, with highest mortality in the smaller seed. Prevalence and intensity of *V. aestuarianus* infection transiently decreased in oysters exposed to UV-B. Prevalence and intensity of *V. aestuarianus* infection were most reduced in oysters submerged in seawater during the UV exposure treatment. Thus, UV-B caused more *C. gigas* death, despite a decrease in *Vibrio* infection. The data identify a causative factor for, thus far, unexplained mortality events during the summer. These findings will inform husbandry management of this commercially important species.

## 4.2 Introduction

Bivalve aquaculture is a significant contributor to global food supply, and helps meet the increasing food requirements of a rapidly growing world population (FAO, 2018). The interest in extractive species such as bivalve molluscs and seaweeds is increasing – due to their ability to improve water quality through filtration and remediation (FAO, 2018), and thus their suitability to be cultured as part of sustainable integrated multitrophic aquaculture. Biosecurity and climate change are the main challenges faced by the aquaculture industry (FAO, 2018). The effects of climate change on pathogen prevalence, distribution, and host-parasite relationships are well documented (Harvell *et al*., 2002, Brooks and Hoberg, 2007, Danovaro *et al*., 2011) with temperature and salinity (Haskin & Ford, 1982, Studer & Poulin,

2012), acidification (Asplund *et al*., 2014) and UV-B radiation (Marcogliese, 2008) being particularly impactful. Alterations in host-parasite relationships may have significant consequences for marine and coastal aquaculture and ecosystems (Cochrane *et al*., 2009, Callaway *et al*., 2012).

Cupped oysters *Crassostrea* spp. are economically significant, compromising roughly 30% of the 17 million tonnes of cultured molluscs in 2016, with a global revenue of 30 billion USD (FAO, 2018). In recent years, oyster production sites around Europe have experienced increased seasonal mortality events, termed 'summer mortality' (European Food Safety Authority [EFSA], 2015). Pacific oyster *Crassostrea gigas* summer mortality episodes have occurred sporadically in numerous regions, first recorded in Japan in 1915 (Takeuchi *et al*., 1960) and later in the USA in the 1950s (Glude, 1975). More recently, *C. gigas* cultivation sites in France, UK and Ireland have been increasingly affected by summer mortality of oyster spat from the early 1990s and 2000s (Lacoste *et al*., 2001, Segarra *et al*., 2010, Garcia *et al*., 2011, Lynch *et al*., 2012, Renault *et al*., 2014). Abiotic factors alone are not enough to induce the high rate of mortality experienced by oyster hatcheries and production sites (Petton *et al*., 2015). Rather, a complex multifactorial aetiology involving environmental factors and pathogens has been suggested as the driver of these mortality events (EFSA, 2015). There are many abiotic stressors associated with inter-tidal habitats. Some of these environmental factors such as extreme temperatures and high salinity are known to play a role in oyster mortality events (Malham *et al*., 2009, Barbosa Solomieu *et al*., 2015). The viral pathogens most commonly associated with summer mortality events are ostreid herpes virus-1 (OsHV-1), and a newly described variant ostreid herpes virus-1 microVar (OsHV-1 µVar), both of

which were linked with numerous mortality events in France (Segarra *et al*., 2010, Garcia *et al*., 2011, Renault *et al*., 2014), USA (Burge *et al*., 2006), and in Ireland (Lynch *et al*., 2012, Peeler *et al*., 2012). Species of the ubiquitous marine bacteria *Vibrio*, including *V. splendidus* and *V. aestuarianus* have also been linked to mass mortality events in *C. gigas* (EFSA, 2015). *V. aestuarianus* has been present in oysters and in the environment around French coasts since 2001, but the detection frequency has increased markedly since 2012 (Garnier *et al*., 2007, Saulnier *et al*., 2010, Lemire *et al*., 2015). *V. aestuarianus* was first detected in Ireland in *C. gigas* samples collected between 2008 to 2012, across all host age groups, and reaching 100% prevalence in some groups. The presence of *V. aestuarianus* often co-occurs with OsHV-1 infection (EFSA, 2015).

Numerous past studies have investigated the role of temperature, salinity, and acidification in oyster mass mortalities (Petton *et al*., 2015, Dubert *et al*., 2017). However, to date, no studies could be found which explored the role of UV-B in these mortality events. Given the seasonality of mortality, the intertidal habitat in which oysters are primarily cultured, and the documented impacts of UV-B on a range of hosts and pathogens, a role for solar UV-B radiation in oyster mass mortality events can be hypothesised. Furthermore, there is good evidence that ambient UV-B levels are changing. Climate change can have a substantial influence on surface UV levels through alterations in temperature, greenhouse gasses (GHGs) and aerosols concentrations, and especially cloud cover (Bais *et al*., 2018). In aquatic environments, future trends in UV-B surface and sub-surface levels have proven particularly difficult to predict given the impact of acidification, salinity, temperature, and dissolved organic matter content on sub-surface

UV-B levels (Hader *et al*., 2015, Bais *et al*., 2018). Finally, there is a knowledge gap concerning direct effects of UV-B on the health of bivalves (Kett *et al*., 2020). UV can cause DNA degradation and haemocyte apoptosis, along with other morphological alterations, in bivalve cells (Gervais *et al*., 2015). UV-B can also inhibit fertilisation in bivalves as reported for zebra mussels (*Dreissena polymorpha*) (Seaver *et al*., 2009), and increase mortality rates in molluscs including the false limpet *Siphonaria denticulata* and the littorinid *Bembicium nanum* and other marine invertebrates (Przeslawski *et al*., 2005, Llabrés *et al*., 2013). Although the exact mechanism of UV induced mortality is often unreported, literature suggests that DNA mutations, apoptosis, morphological changes, and oxidative stress, all play a role in the UV induced mortality of aquatic animals (Kouwenberg *et al*., 1999, Gervais *et al*., 2015). Increased UV-B radiation can also cause immunosuppression in host organisms (Hart *et al.*, 2011). At the same time, increased UV-B can negatively affect pathogens and parasites in surface waters including those pathogenic to humans such as the bivalve-associated human pathogens norovirus and *V. cholerae, V. parahaemolyticus* (Marcogliese, 2001, Harvell *et al*., 2002, Flannery *et al*., 2013, Widmann, 2013). The disinfectant capacity of UV radiation is often associated with shorter wavelength UV-C (□□□□ – 280 nm) (de Alba *et al*., 2021, Fernández-Boo *et al*., 2021) but extends into the UV-B wavelength band.

The aims of this study were to investigate the impact of increased supplemental UV-B radiation on the performance (survival) of Pacific oysters *C. gigas* and on the development of a common bacterial pathogen *V. aestuarianus* in oysters. Supplemental UV-B was intended to represent high

incident UV-B radiation in a shallow water column (Trial 1: High intensity, underwater trial) or increased atmospheric UV-B during tidal emersion (Trial 2: Low intensity, emersion trial). Findings from this study will enhance understanding of host-pathogen dynamics in inter-tidal and coastal habitats and will strengthen the ongoing global investigation into the multifaceted drivers of *C. gigas* summer mortality syndrome.

## 4.3 Materials & Methods

### 4.3.1 Sample collection site

The experiments used oysters of three size classes (Table S1; Supplementary material). 'Small' and 'medium' sized seed oysters were sourced from oyster producers in Dungarvan Bay (Dungarvan Shellfish Ltd.), Co. Waterford (52º 3' 54" N, 7º 35' 29" W) where they were reared in a nursery and re-laid in the commercial production field site. All seed oysters were collected from the on-site nursey in late June of 2018 and housed in acclimation tanks and experimental trials were conducted in July and August 2018. 'Large seed' sized oysters (Table S1) used in the HI Trial were sourced from Castlemaine Harbour, Co Kerry (52º 7' 4" N, 9º 52' 43"). Both sites are known to have recurring oyster mortality episodes associated with OsHV-1 µVar and *Vibrio aestuarianus* as confirmed by the national reference laboratory (NFL) for molluscan diseases (Marine Institute, Ireland [EFSA. 2015]).

### 4.3.2 UV-B treatment

UV-B emitting lamps were sourced from Atlantium technologies Ltd., Israel. Sodium acetate filters were used to screen out short wave radiation (<280 nm) and pure cotton muslin cloth was used to reduce output intensity of bulbs. Full spectrum irradiance was measured with a spectroradiometer (Ocean

Optics, USB2000+). UV radiation emitted by the bulbs was largely within UV-B wavelengths, primarily 305 nm – 315 nm (Figure 1; supplementary material). Total UV Irradiance was measured with a PMA2200 photometer (Solar Light Inc., USA). To get a reading of underwater irradiance experienced by oysters, the total UV irradiance value was recorded beneath the filled tank, from which the 'blank' value (measured beneath an empty tank) was subtracted. Control groups were held in similar aquaria although with a black plastic lid instead of the bulb-fitted lid. Holding and experimental tanks were kept indoors and in the dark to ensure no external UV radiation would affect the study. All tanks were equally and sufficiently stocked with phytoplankton feed (5 ml standard aquarium algae feed in 20 L seawater on alternate days) and kept under identical environmental conditions.

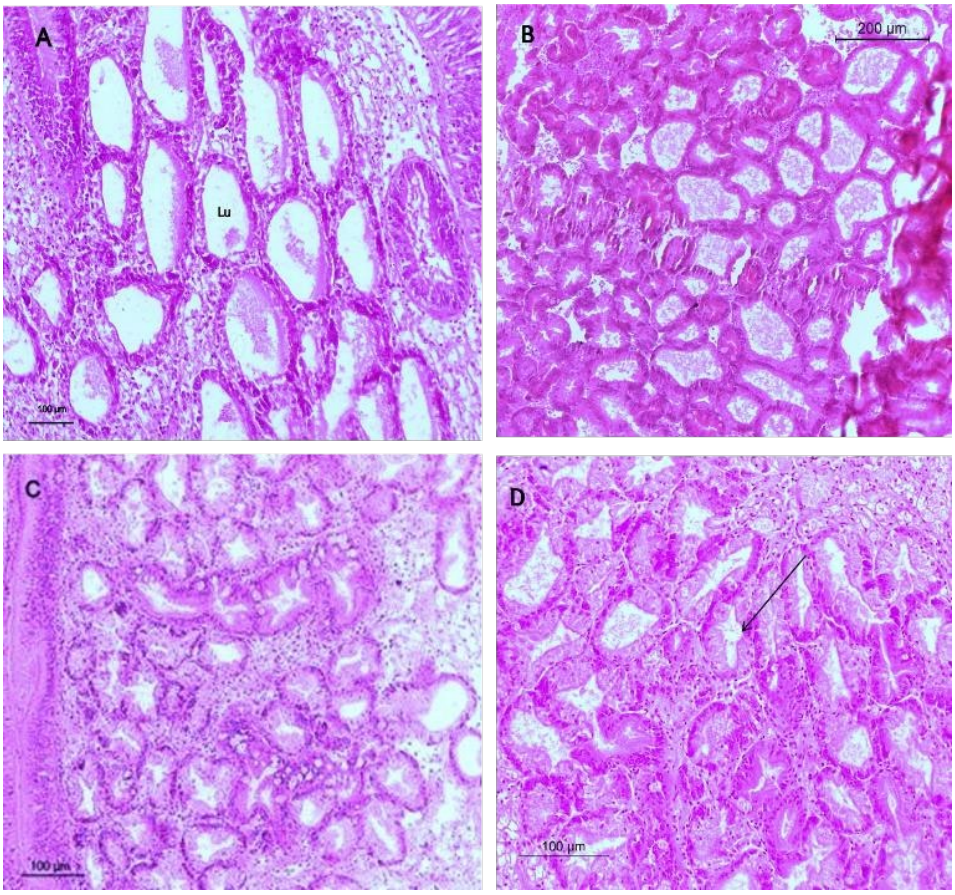



Figure 1. Digestive gland atrophy scored as A: 4 with dilated lumen (Lu), B:

3, C: 1, and D: 0 with 'normal' digestive gland wall thickness (arrow). As classified in Kim *et al*., 2006. Detailed description in Table S2-Supplementary material. H&E stain.

### 4.3.3 Trial 1: High intensity (HI), underwater trial

The 'high intensity underwater trial' was designed to investigate the impact of short duration, high intensity UV-B exposure on the health of different sized oyster seeds and on their parasite load. The high intensity does was 0.254 mW/cm$^2$ for 4 minutes 35 seconds to achieve a total dose of 0.7 kJ/m$^2$ over three consecutive days (Atlantium Technologies Ltd., Israel). The total dose of 0.7 kJ/m$^2$ is equivalent to a full afternoon dose on a cloudless Spring or Autumnal day in southern Ireland (Met Eireann Observatory, Pers. Comm.).

The high intensity, underwater trial analysed the performance of 700 oyster seed: 250 'small seed', 250 'medium seed', and 200 'large seed' oysters. After an initial 5-day acclimation period at 18 $^{o}$C, an initial sample (n = 30 per size class) was taken and screened for pathogen prevalence. The remainder of the oysters were split among three experimental and three control tanks with 30 oysters of each size class in each tank (total N per tank = 90). All animals were submerged in artificial seawater (ASW) for the duration of the experiment. UV was administered over 3 days (Days 1 – 3) starting at noon each day, followed by a recovery period lasting 8 days (Days 4 - 11). Post-treatment oysters were returned to phytoplankton (feed) stocked holding tanks of the same size and density, which were kept in the dark to reduce effects of external light. During the UV treatment and subsequent recovery periods, mortality was monitored daily. Moribund animals were removed immediately, and oysters were screened for the presence of any

pathogens. Individuals were defined as moribund/dead when the oyster shell was gaping and unresponsive to stimuli (tapping of shell) i.e., did not close fully or tissue was absent from the shell. Due to the small size of the seed oysters, some moribund individuals were unable to be screened (n = 12). Samples for histological and PCR pathogen screening were taken daily during the recovery period. Three oysters were randomly sampled from each of the three replicate tanks from both control and experimental groups (n = 9/group at each sampling period) for each of the three oyster sizes.

### 4.3.4 Trial 2: Low intensity (LI), emersion trial

The 'low intensity emersion trial', was designed to assess the impact of a typical three-hour UV-B exposure on a clear summer day in the south of Ireland during low tide. Exposure to UV-B was 0.0064 mW/cm$^2$ for 180 minutes per day for three days for a total dose of 0.7 kJ/m$^2$ daily. Oysters were aerially exposed throughout the duration of the exposure treatment (180 min/day) and subsequently returned to phytoplankton (feed) stocked holding tanks kept in the dark. The aerial LI trial assessed the performance of 250 'small seed' and 250 'medium seed'. The 'small seed' oysters were larger in size than the 'medium seed' in HI Trial but were considered small relative to the "medium seed" oysters used in this trial.

Initially, 500 oysters were held in 50-litre ASW incubation tanks for 10 days at 20 ºC. Following the incubation period, an initial sample (n = 30 per size class) was screened for pathogens. The remainder were split among three experimental and three control tanks with 30 oysters of each size class in each 20-litre tank (total N per tank = 60).

The exposure treatments were administered over 3 days followed by a 7-day recovery and monitoring period. Following the treatment, oysters were returned to adequately stocked phytoplankton (feed) holding tanks of the same size and density (5 ml standard aquarium algae feed in 20 L seawater on alternate days), which were kept in the dark to reduce effects of external light. Mortality was monitored daily throughout the experiment and moribund animals were removed immediately. Due to the small size of the seed oysters, some moribund individuals were unable to be screened (n = 30). During both the exposure and recovery periods, three oysters were randomly sampled from each of the three replicate tanks in both control and experimental groups (n = 9/group at each sampling period) for histology and molecular pathogen screening. Sampling was conducted before ('Day *n* (a)') and after ('Day *n* (b)') each of the three UV-B exposure treatments (Days 1 – 3) and once during the recovery days (Day 4, 5, 6, and 10) after the initial exposure treatment.

### 4.3.5 Screening of oysters

Before dissection of the sampled oysters, weight and length of the oysters were recorded. Weight (g) was taken wet (shell plus tissue) with a digital balance scale. Length (mm) was measured with a vernier callipers.

### 4.3.6 Screening of tissue sections for morphological changes

A 5 mm cross-section of oyster tissue was spliced from the posterior of the labial palps and this contained sections of gill, mantle, visceral mass, gonad, and digestive organs (Ifremer, 2011). These were fixed in Davidson's solution and paraffinized with a Citadell processor (Brand) following methods in Ifremer (2011), before being sectioned at 5 µm, placed onto microscope slides and dehydrated at 40 °C overnight. The slides were deparaffinised and stained

with haematoxylin and eosin (H&E) stain according to Humanson (1979) prior to being examined using light microscopy. Common pathologies were recorded including digestive gland atrophy scored on a semi-quantitative scale as per Kim *et al*. (2006) (Table S2; supplementary material), haemocytic accumulation i.e., infiltration of haemocytes usually associated with inflammation, tissue necrosis i.e., cell death and tissue damage, and deciliation i.e. the destruction of cilia usually on the gill or intestinal lining. For histopathological analysis, samples from both trials were pooled together.

### 4.3.7 PCR, QPCR and *in situ* hybridisation (ISH) screening of OSHV-1 uvar and *V. aestuarianus.*

A small (2 $mm^2$) piece of gill and external tissues was excised from each oyster for DNA extraction. Extraction was carried out using the Chelex-100 method (Walsh *et al*., 1991, Lynch *et al*., 2012). PCR was used to screen for OsHV-1 µVar using OHVA/OHVB primer pairs (Lynch *et al*., 2013). Quantitative PCR (qPCR) was used to confirm PCR results using HVDP-F and HVDP-R primers (Ifremer, 2011). *Vibrio aestuarianus* was screened by qPCR with TaqMan® MGB methodology as described in McCleary & Henshilwood (2015) with a $C_t$ cut-off point of 37 with any values above this deemed negative for presence of *V. aestuarianus*. Intensity of infection was categorised according to the cut-off point i.e., low infection: $C_t$ 35 - 37, medium infection: $C_t$ 30 – 35, high infection $C_t$ <30.

*In situ* hybridisation (ISH) for *Vibrio* spp. was carried out to assess the tissue distribution of generic *Vibrio* species according to Kett *et al*. (2022) Oyster tissue samples were fixed and sectioned as for histological examination and placed onto silane-prep slides. For ISH, a digoxigenin-labelled probe was used, as described in Lynch *et al*. (2010) but modified for optimisation

following supplier guidelines (www.abcam.com) and previous work in Russell & Sambrook (2001) and Lynch *et al*. (2010).

### 4.3.8 Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics software version 24. Survival analysis was carried out using Kaplan-Meier estimator to compare log-rank values using treatment group as the comparison factor, this was performed using XLSTAT (Addinsoft, 2022). For each size class, 2 x 2 Chi squared tests of independence were used to test for differences and prevalence and intensity of *V. aestuarianus* infection. Replicate tanks were pooled.

## 4.4 Results

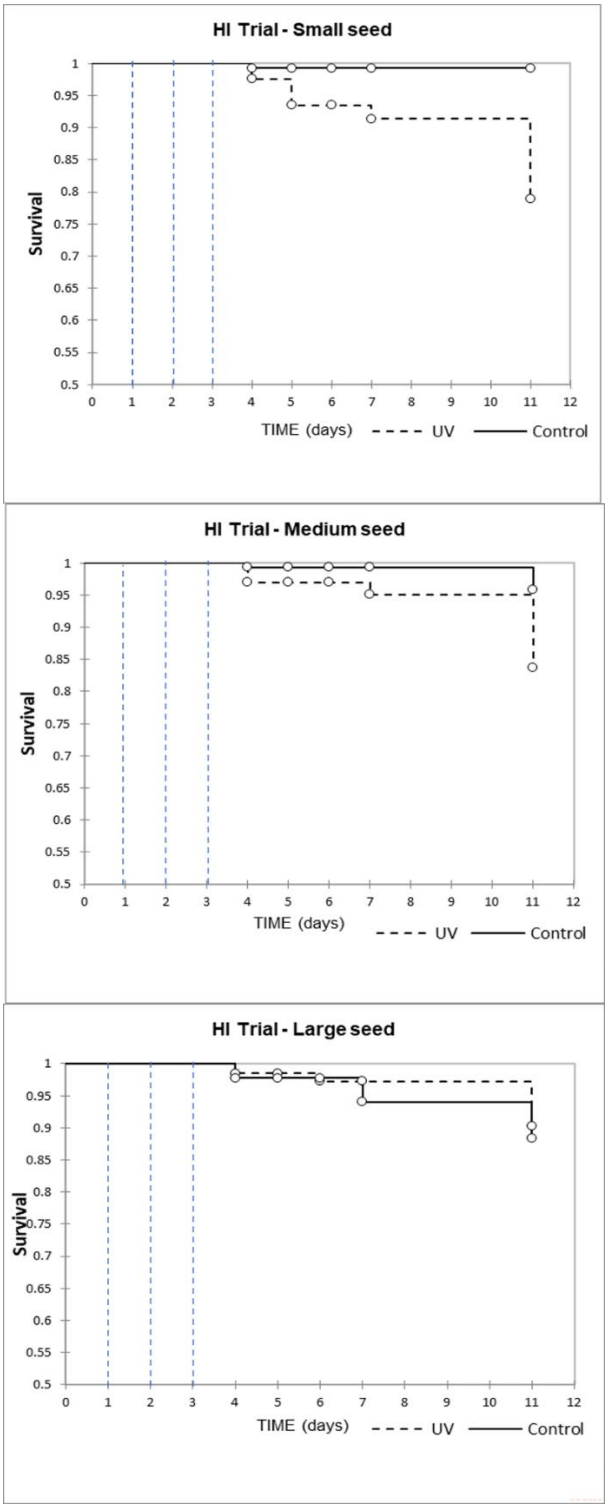
### 4.4.1 High intensity (HI) underwater trial

#### *4.4.1.1 Oyster Survival*

Oyster survival throughout the HI trial is shown in Kaplan-Meier plots in figure 2. Mean survival time was significantly higher in the 'small seed' control group compared to their UV exposed counterparts (Log-rank test value: 10.8, df = 1, p = 0.001). Similarly, survival was significantly higher in control 'medium seed' groups compared to their UV exposed counterparts (Log rank value: 4.5, df = 1, p = 0.034). Survival of the 'large seed' groups was not found to be significantly different (Log-rank value: 0.054, df = 1, p = 0.8). Oyster survival in any group was not affected during the UV exposure treatment period, as indicated by the blue vertical broken lines in figure 2.

Survival did not change in the control groups of the 'small seed' and 'medium seed' cohorts throughout the entire trial. Survival of the UV exposed 'small seed' decreased to 93% by 'Day 5' and to 78% by the end of the experiment on 'Day 11'. In the UV exposed 'medium seed' group, survival decreased to

95% by ‘Day 7’ and to 83% at the end of the experiment. In the ‘large seed’ group, survival at the end of the experiment was 88% and 90% in the control group and the UV exposed groups respectively.




1

2 Figure 2. Oyster survival in ‘small seed’(top), ‘medium seed’ (middle), and

3 'large seed' (bottom) in HI Trial either exposed to UV-B (broken line) or kept

4 under control conditions (solid line). Sampling period is shown in days after

5 the initial sample. Vertical blue lines represent UV exposure treatments.

6 Circles show censoring times when oyster mortality was counted.

#### *4.4.1.2 Pathogen screening.*

*OsHV-1 µVar*

A total of 220 oysters (n = 110 each of control and UV exposed, sampled evenly from size groups across time periods) were tested for OsHV-1 µVar using PCR. No OsHV-1 µVar was detected in any oyster throughout the HI trial. The absence of OsHV-1 µVar was further confirmed by qPCR with a total of 120 oysters (n = 60 each of control and UV exposed), with no positive detection (i.e., Ct: <37).

*Vibrio aestuarianus*

*V. aestuarianus* was detected in oysters when they were first brought into the lab. *V. aestuarianus* was detected in the initial sample with 100%, 90% and 40% of individuals positive in the 'small', 'medium' and 'large' size groups, respectively (Figure 3). Overall, throughout the recovery period from 'Day 4' to 'Day 11', infection prevalence was lower in the UV exposed groups compared to control groups. Mean infection prevalence throughout this period for the UV exposed small seed, medium seed and large seed was 56.5%, 38.2% and 32.2% respectively. Comparative infection prevalence in the control groups for this period were 84.6%, 86.4% and 53.8% respectively. On 'Day 5', 48 hours after the final UV-B exposure treatment, infection prevalence had significantly decreased by 87.5% in the UV exposed 'small

seed' group compared to the same group on 'Day 4' ($\chi^2$ = 13.38, df = 1, p < 0.001, N = 17) (Figure 3 C). *V. aestuarianus* had also decreased in the UV-B treated 'medium seed' by 68%, although this was not found to be significant ($\chi^2$ = □□□□ df = 1, p = 0.011, N = 19) (Figure 3 B) and significantly in the 'large seed' group by 40% ($\chi^2$ = □□□□□ df = 1, p = 0.031, N = 17) (Figure 3 A).

In the control groups within the same timeframe, infection prevalence did not show such dramatic decreases. Over the next 6 days of the recovery period (Days 6, 7 and 11), infection prevalence did not differ significantly between UV exposed and control treatments within any size group. At the end of the Trial 1, infection prevalence in the large seed size group had returned to preexposure levels and was not significantly different to the control group at that time. In the small and medium size groups, at the final time point in the trial, infection prevalence was significantly lower than the initial (pre-exposure) infection levels in the UV exposed small seed ($\chi^2$ = 4.5, df = 1, p = 0.034, N = 21) and medium seed groups ($\chi^2$ = 4.55, df = 1, p = 0.032, N = 19). This effect was not observed in the UV exposed large seed group ($\chi^2$ = 0, df = 1, p =1, N = 25).

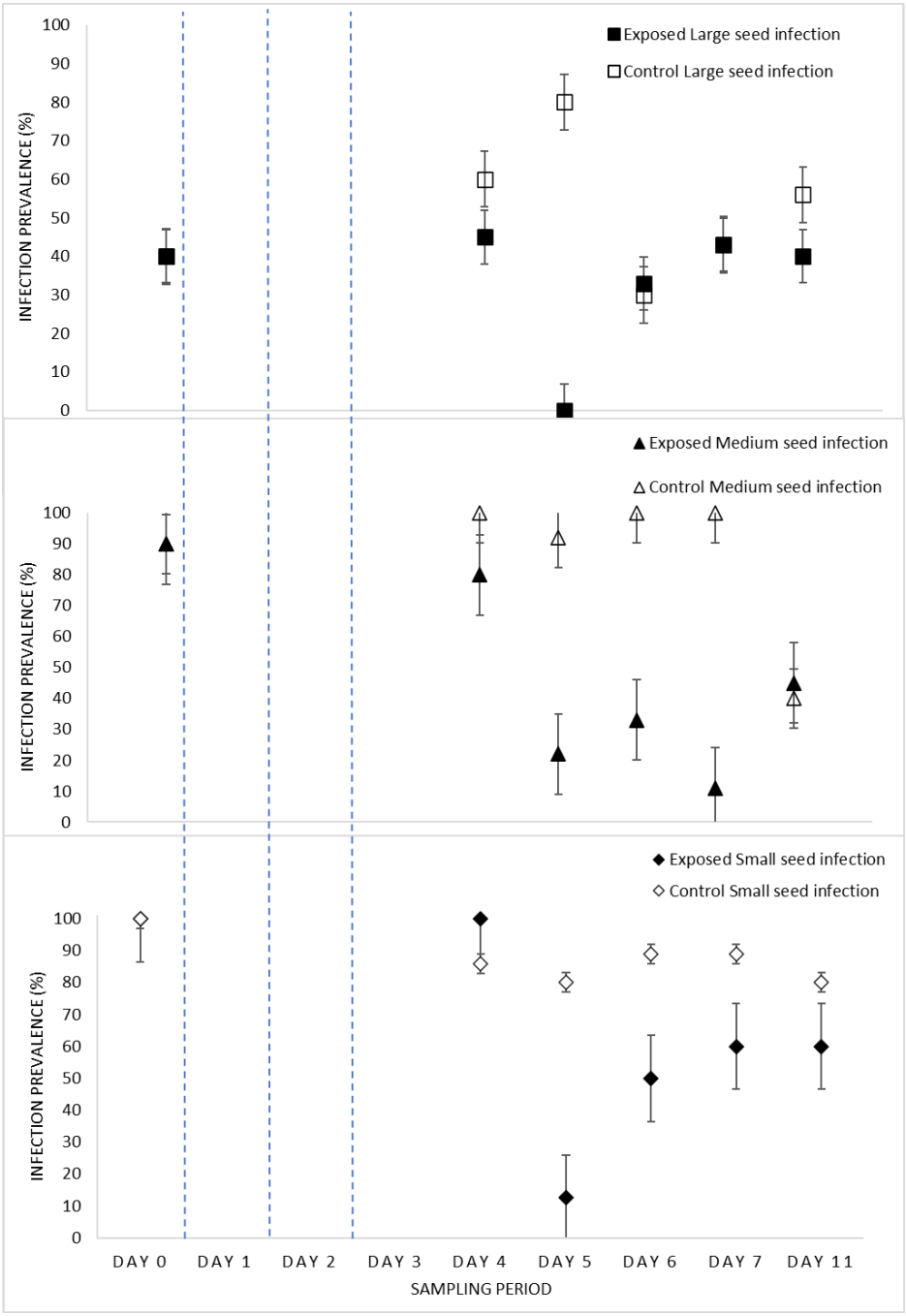



Figure 3. Mean *V. aestuarianus* infection prevalence as a function of UV-B exposure, in **A**: 'large', **B**: 'medium', and **C**: 'small' seed groups in HI Trial. Vertical blue broken lines represent UV-B exposure treatments. N = 9 oysters per marker. Day 0 N = 30 per size group. Error bars represent standard error.

To measure *V. aestuarianus* infection intensity in 324 oysters (control: n = 52/size class, UV exposed: n = 46/size class, initial sample: n = 10/size class)

qPCR was used. Some moribund oysters could not be screened due to degradation of tissues (control: n = 15, UV exposed: n = 22). In all sampled oysters in the high UV intensity underwater trial, the intensity of infection was reduced in groups exposed to UV-B compared to control groups (Figure 4). For example, on 'Day 5' (48 hrs post exposure), 24 (63%) of 38 individuals in the control group were classed with 'high infection' (Ct: < 30) while nine (23.5%) of this cohort were classed with 'medium' infection (Ct: 30 – 35) and four (10.5%) individuals from this group were 'uninfected' (Ct: >37). In comparison, in the UV-B exposed group sampled at 'Day 5' (n = 25), no animals were classed as having either 'high' or 'medium' infection level, while 19 (76%) individuals were uninfected.

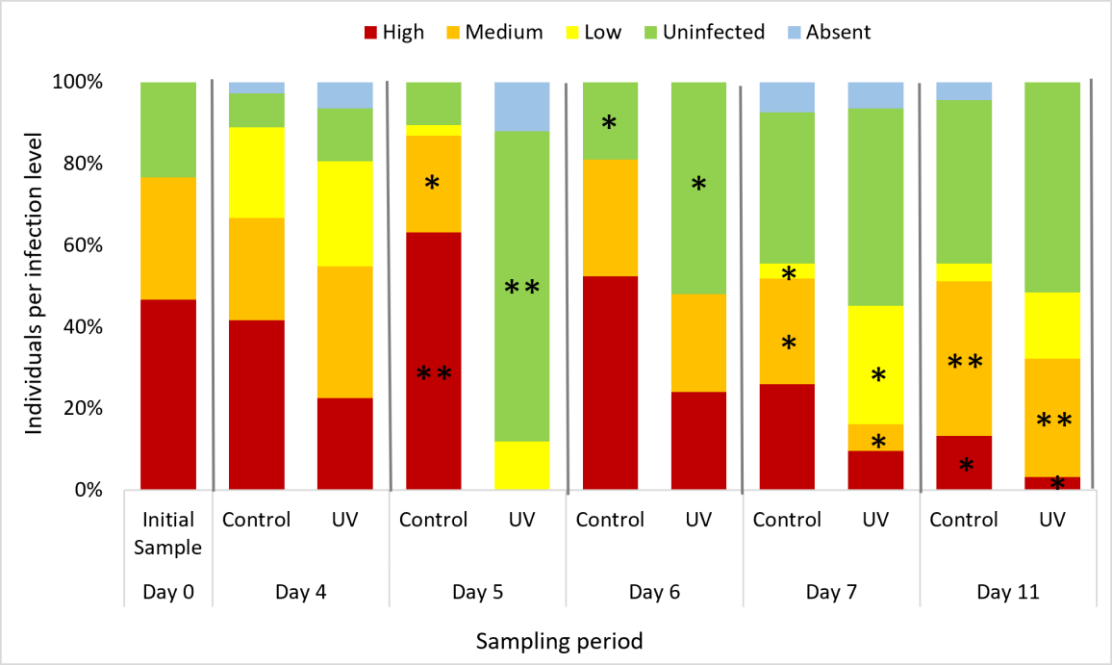



Figure 4. *V. aestuarianus* infection intensity in live- and moribund-sampled, UV exposed and control oysters throughout the HI trial. Statistical analysis compared number of individuals per infection class with the same data on previous sampling point, * denotes $p < 0.05$, ** denotes $p < 0.001$.

### 4.4.2 Low Intensity (LI) aerial exposure

#### *4.4.2.1 Oyster Survival*

Oyster survival in the LI trial is shown on Kaplan-Meier plots in Fig. 5. Survival was significantly lower in UV exposed 'small seed' compared to their control counterparts (Log-rank: 15.12, df = 1, $p < 0.0001$). Similarly, in the 'large seed' groups, survival was significantly lower in those exposed to UV compared to control groups (Log-rank: 16.74, df = 1. $P < 0.0001$). Oyster survival in both size groups did not decrease during the UV exposure period as indicated by the blue vertical broken lines in Fig. 5. The change in oyster survival was observed on 'Day 5' 72 hrs after the final UV exposure. At this time oyster survival decreased from 95% to 81% in the 'small seed' group, and from 95% to 87% in the 'large seed' group. At the end of the experiment on 'Day 9', 'small seed' survival in the control group was at 88% compared to 67% in the UV exposed group. In the 'large seed' group at the end of the experiment, oyster survival was observed to be 93% in the control group compared to 72% in the UV exposed group (figure 5).

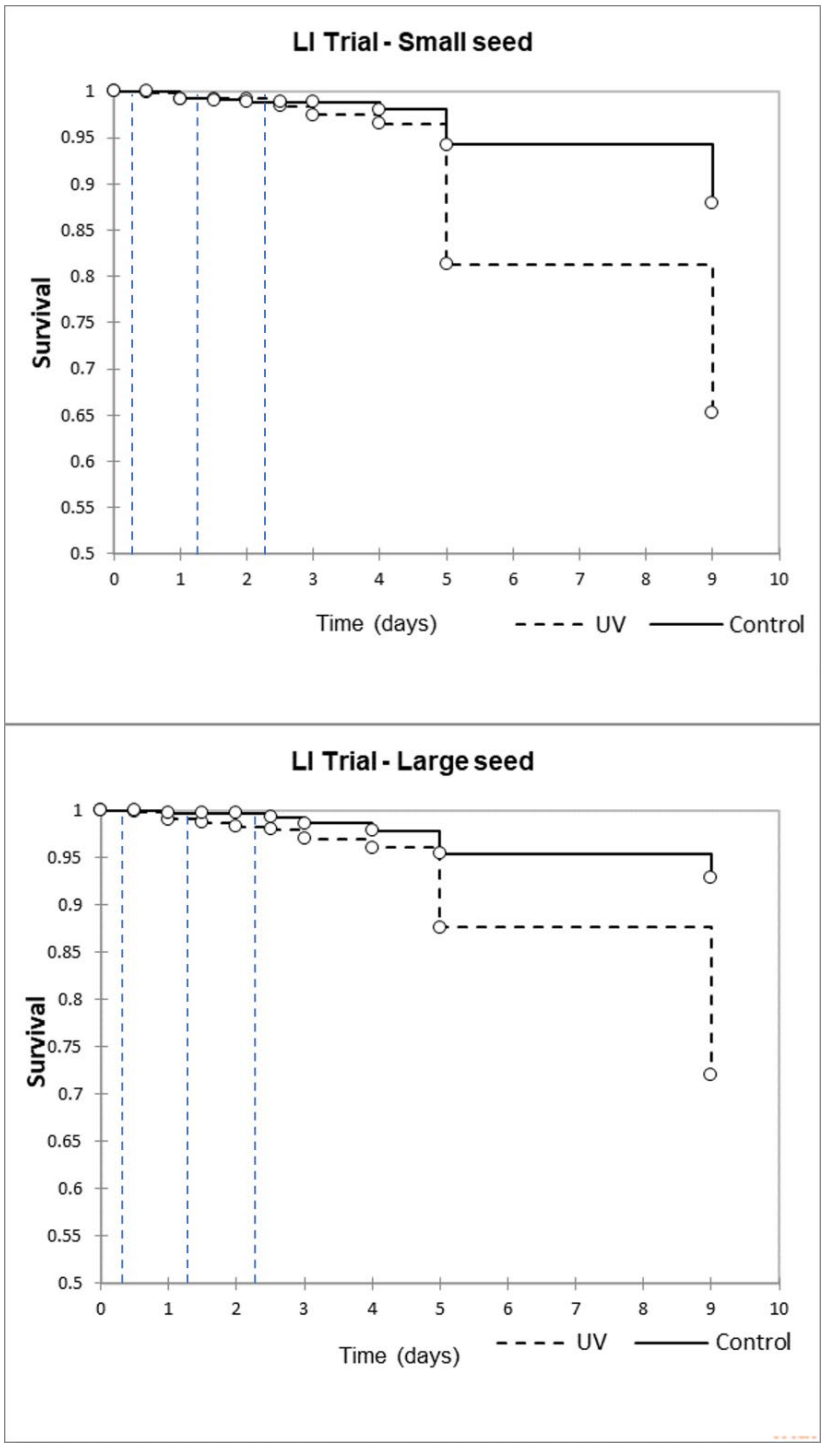



Figure 5. Oyster survival throughout the LI Trial either exposed to UV-B (broken line) or kept under control conditions (solid line). Sampling period is shown in days after the initial sample. Vertical blue lines represent UV exposure treatments. Circles show censoring times when oyster mortality was counted.

#### 4.4.2.2 Pathogen screening *OsHV-1 µVar*

A total of 230 oysters (n = 115 each of control and UV exposed groups, evenly split across size groups and sampling periods) were screened for OsHV-1 µVar with PCR and no positive detection was observed. This finding was confirmed by qPCR screening of 100 oysters (n = 50 each of control and UV exposed groups, evenly split across size groups and sampling periods)

*Vibrio aestuarianus*

At the start of the trial *V. aestuarianus* was prevalent in 96% of the 'small seed' oysters and 93% of the 'medium seed' oysters (Figure 6). Overall, throughout the exposure and recovery periods, mean infection prevalence for the UV exposed small and medium size seed were 95.1% and 92.8% respectively. Mean infection prevalence for the small and medium seed control groups were 97% and 96.2% respectively. Following the first day UVB exposure treatment, prevalence of *V. aestuarianus* in 'small' seed exposed to UV-B fell by 35% ($\chi^2 = 18.5$, df = 1, $p < 0.001$, N = 45) and in 'medium' sized seed by 55% ($\chi^2 = 7.5$, df = 1, $p = 0.005$, N = 39). In the control group, infection prevalence increased by 4% in both size groups during the same period ($\chi^2 = 1.8$, df = 1, $p = 0.17$, N = 39). Prior to the next exposure period, infection prevalence returned to pre-exposure levels in all groups ('Day 2').

Also, following the second exposure treatment ('Day 2(b)'), infection prevalence fell by 12% and 10% in the 'small' and 'medium' size UV exposed seed, although not significant, while the control groups remained constant at 100% infection prevalence in both size groups. Following the third and final exposure period ('Day 3(b)'), infection prevalence remained at 100% in all groups.

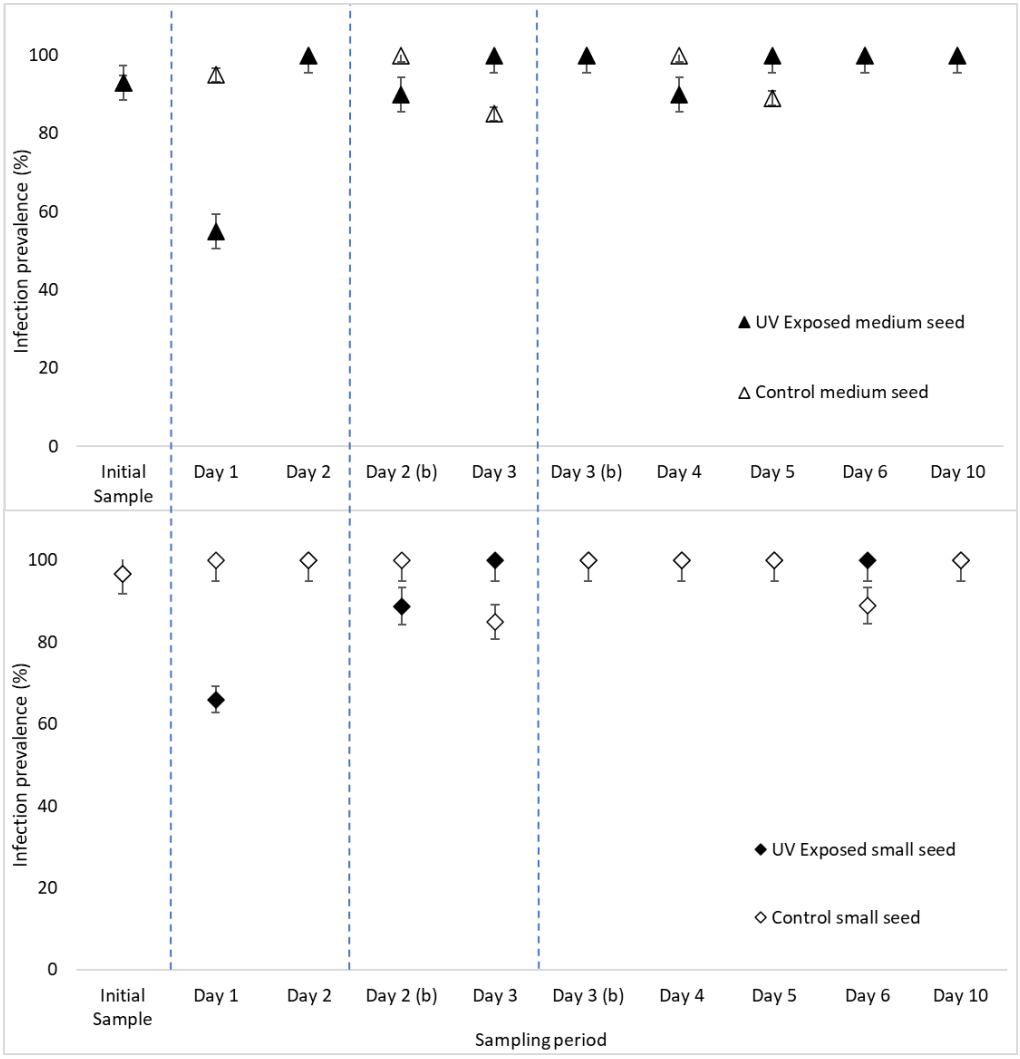



Figure 6. Mean *V. aestuarianus* prevalence of infection in **B:** 'small seed' and **C**: 'medium seed' of UV-B exposed (full icon) and control (empty icons) in the LI Trial. Broken vertical lines represent the subsequent UV-B exposure treatments. Error bars represent standard error.

A total of 451 oysters were removed for *V. aestuarianus* screening during the trial, 143 control and 175 UV-exposed animals and 67 pre-trial animals. Included in these numbers were 34 control and 32 UV exposed moribund oysters removed from experimental tanks. Some of these could not be screened due to degradation of tissues. Pathogen infection intensity data were collected from 161 control oysters and 193 UV exposed oysters (Figure 7).

Overall, UV exposed groups had lower infection intensity during the exposure period compared to control cohorts. As the LI Trial progressed into the recovery period (days following UV exposure), infection intensity increased in all groups with fewer uninfected individuals at the end of the trial. On 'Day 1', immediately following the first UV exposure treatment, 4 of the 20 (20%) individuals in the control group exhibited a 'high' degree of *V. aestuarianus* infection, while none of the 18 (0%) UV exposed sampled at the same point showed 'high' infection levels. The following morning 'Day 2 (a)' before the subsequent UV exposure, 'high' *V. aestuarianus* infection levels were detected in both control (69%) and UV exposed groups (90%).

Following the second UV exposure 'Day 2(b)', the number of 'high' infected individuals dropped in both groups although these decreases were not statistically different. The number of oysters with 'high' infection levels in the UV exposed group on 'Day 3 (a)' (n = 14) was significantly higher compared to immediately following the treatment 'Day 3(b)' (n = 3), which was not observed in the control group before (n = 1) and after treatment (n = 3) ($\chi^2 = 5.2$, df = 1, p = 0.02, N = 21).

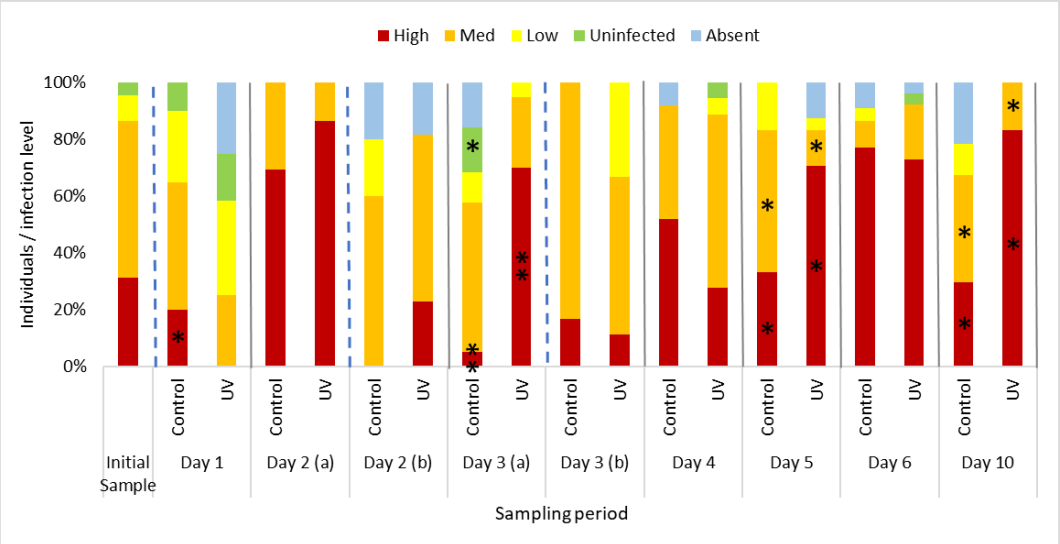


Figure 7. *V. aestuarianus* infection intensity of live- and moribund-sampled individuals from control and UV-B exposed groups of both size groups combined throughout LI Trial. Statistical analysis compared number of individuals per infection class with the same data on previous sampling point; * denotes p =/< 0.05, ** denotes p < 0.005. Broken blue lines represent UV exposure treatments.

*In situ* hybridisation was carried out to confirm the presence of *Vibrio* bacteria on control (n = 6) and UV exposed (n = 6) oysters from the LI Trial. All oysters which exhibited positive *V. aestuarianus* infection *via* qPCR, showed positive staining of *Vibrio* cells (Fig.8).

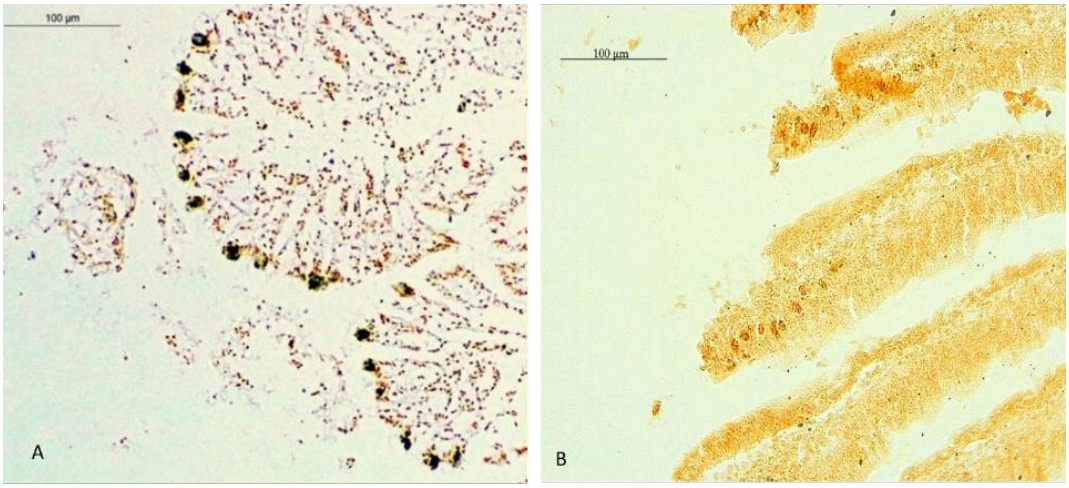



Figure 8. **A:** Example of ISH of *C. gigas* tissue showing positive *Vibrio* infection around gill tissue. **B:** ISH of *C. gigas* gill tissue with no detection of *Vibrio* bacteria.

### 4.4.3 Histological examination

All observed pathologies were associated with *V. aestuarianus* infection, in both control (n = 15) and UV exposed groups (n= 15) split evenly amongst size groups. Haemocyte accumulation, tissue necrosis and digestive gland

atrophy, as described and shown in Kett *et al*. (2022), were more frequent and pronounced in the oyster seed with 'high' intensity of *V. aestuarianus* infection (Table 1). None of the major pathologies observed were recorded in uninfected oysters (n = 6). Groups of small, dark-staining, bacteria-like cells were seen in tissues in two of the five (40%) oysters with a high infection level from the control group (n = 5). These cell-clusters were not observed in oysters with a high infection level from the UV exposed group (n = 2), nor in 'medium' infected and uninfected seed from the control or UV exposed group.

1 2 **Table 1. Pathology associated with oyster seed in various levels of *V. aestuarianus* infection.**

| ID | Treatment | *V. aes.* infection level | Ct value | Digestive gland atrophy | Haemocyte accumulation | Necrosis | Deciliation |
|---|---|---|---|---|---|---|---|
| **U1** | Control | Uninfected | 36.8 | 0 | - | - | - |
| **U2** | UV Exposed | Uninfected | 37.1 | 0 | - | - | - |
| **U3** | UV Exposed | Uninfected | 37.1 | 0 | - | - | - |
| **U4** | UV Exposed | Uninfected | 36.9 | 1 | - | - | - |
| | | | | | | | |
| **M1** | Control | Medium | 33.0 | 1 | - | - | Present |
| **M2** | Control | Medium | 34.0 | 3 | - | - | - |
| **M3** | Control | Medium | 30.1 | 0 | - | M, G | Present |
| **M4** | UV Exposed | Medium | 33.7 | 1 | Present | - | - |
| | | | | | | | |
| **H1** | Control | High | 28.7 | 2 | Present | - | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **H2** | Control | High | 29.7 | 3 | Present | CT, M | Present |
| **H3** | Control | High | 17.4 | 3 | - | CT | - |
| **H4** | Control | High | 28.9 | 2 | - | - | Present |
| **H5** | Control | High | 21.1 | 1 | Present | - | - |
| **H6** | UV Exposed | High | 18.5 | 3 | - | CT | - |
| **H7** | UV Exposed | High | 19.6 | 4 | - | - | Present |

M = Mantle, G = Gill, CT = Connective Tissue.

## 4.5 Discussion

This study shows that UV-B radiation decreases Pacific oyster *Crassostrea gigas* survival, particularly in younger cohorts that have thinner and more transparent shells. In addition, UV exposure transiently decreased *V. aestuarianus* prevalence and infection intensity in different oyster age groups.

### 4.5.1 UV-B induced mortality

UV-B exposure was associated with decreased oyster survival i.e. increased mortality. The mechanism underlying such mortality may be linked to the genotoxic effects of this type of radiation causing genetic and histological damage beyond functionality (Gervais *et al*., 2015, Kett *et al*., 2020). UV exposure will be most pronounced at lower latitudes, but also in shallower waters, especially if these are UV transparent and have low organic matter content. Thus, UV-B exposure should be considered when positioning oyster beds in the intertidal region. It was noted that smaller individuals experience the highest rates of UV-B mortality, and this is most likely due to their thin

shells which are potentially more transparent to stressors including UV radiation (Davis *et al*., 2013, Llabres *et al*., 2013). Overall, survival was lowest when oysters were exposed to a relatively low daily dose of UV-B, but while being out of water. It could be speculated that oysters will close up once removed from the water column and should be relatively UV-protected. In fact, it had been expected that the submerged oysters in the high intensity trial would have been most UV affected, as these oysters were agape, resulting in a potential opening for easy penetration of UV wavelengths. However, this was not the case, although the intensity and duration of the exposure are contributing factors to this finding. Lacking understanding of UV penetration through bivalve shells, together with potential impacts of the combination of two stressors, drought and UV-B currently limit the ability to predict the scale of UV-induced mortality.

### 4.5.2 UV-B impacts on infection levels

In both trials, exposure to UV-B resulted in transiently lowered levels of *V. aestuarianus* in oysters. It could be argued that the transient decrease in *V. aestuarianus* prevalence was linked to the mortality of infected oyster seed and that survivor screening bias occurred. However, the intensity of infection in moribund samples did not reflect this. In fact, the majority of moribund oysters exposed to UV-B also showed relatively low infection levels. Thus, the decrease in infection prevalence was not due to the specific die-off of diseased oysters.

These data show that UV-B radiation reduces the number of living bacterial cells in UV exposed oyster tissues. It appears that following UV-B exposure, remaining bacteria in inner oyster tissues, or in holding tanks, were reproducing at a rapid rate resulting in a measurable increase in bacterial cell

numbers prior to the subsequent exposure. The growth rate of *Vibrio parahaemolyticus* in live oysters *Crassostrea rivularis* is 7.9 and 5.1 log cfu/g after 60 hours when stored at 25 ºC and 15 ºC respectively (Wang *et al*., 2018). This indicates that *Vibrio* can potentially repopulate overnight within the oyster and in the nutrient-fed holding tank. The observed difference in infection prevalence between the two trials may be explained by the fact that in the high intensity trial, the oysters were likely agape and filtering water while submerged, and therefore exuding bacteria into the irradiated water system which could have inactivated the bacteria. In contrast, in the low intensity trial the oysters were aerially exposed and therefore closed. As such, harboured bacteria were protected from the radiation by residing within oyster tissue, resulting in a higher pathogen load, and a decreased UV-impact.

An alternative explanation for the observed data is that UV-induced CPDs affected the efficiency of *Taq* polymerase in the PCR process (Wellinger & Thoma, 1996). This would result in an underestimation of *Vibrio* spp. prevalence. Using *in situ* hybridisation, no *Vibrio* cells were detected in samples deemed 'uninfected' *via* qPCR, and this included UV-exposed samples. The ISH method used here, utilises a primer with a lower G:C content than the Taqman primer used in the qPCR reaction. ISH is frequently used in the detection of gene sequences following UV exposure (Peccia & Hernandez, 2002, Kimura *et al*., 2004). Thus, it is concluded that *Vibrio* spp. incidence was markedly decreased in UV-exposed oyster samples. These data show that the genotoxic effects of UV-B, so widely exploited for water disinfection, can be successfully used to reduce *Vibrio* incidence in oysters. The application of this discovery is, however, problematic given the significant direct, negative effects of UV-B on oyster mortality. It remains to

be seen whether careful manipulation of UV-doses and wavelengths can be used to impact *Vibrio*, without an effect on the host species. Ostreid herpesvirus-1 (OsHV-1 µVar) was not detected in any oyster sample during these experiments. However, OsHV-1 µVar was detected in a separate cohort of oysters originating from the same field site but sampled at a later period in the summer (August – September) when sea temperatures had increased.

### 4.5.3 Morphological changes associated with infection

Histological assays showed that *V. aestuarianus* infection had a marked effect on internal organs of *C. gigas* from both trials, regardless of UV-B exposure. There was a positive correlation between digestive gland atrophy and intensity of infection. No oysters classed as having 'high' *V. aestuarianus* infection (Ct <30) had 'normal' digestive gland conditions (atrophy score: 0) compared to 75% of the uninfected group exhibiting 'normal' digestive gland conditions. Although atrophy of the digestive glands can also be caused by stressors such as food scarcity or poor nutrition, exposure to contaminants or fluctuations in salinity (Bower *et al*., 1994, Kim *et al*., 2006), in this study, all animals were supplied with equal and adequate food supply. These results indicate a strong correlation between *V. aestuarianus* infection and *C. gigas* digestive gland atrophy. Haemocyte accumulation – a common inflammatory response, necrotic tissue, and deciliation of gills and intestinal lining, were more frequently observed in individuals with 'medium' to 'high' levels of *V. aestuarianus* infection comparted to uninfected individuals.

'Treatment' i.e., UV exposure did not affect the prevalence of pathologies, despite effects on pathogen prevalence, possibly due to the short time frame of the study in which the pathologies would have had time to recover. Yet, it

should be noted that a small sample size was used for the histological examination and a larger sample size may show variations which were not observed in this study. These results demonstrate that *V. aestuarianus* infection has various pathological impacts on *C. gigas* health. These data are useful observations which add to the currently lacking literature on pathological impacts of *V. aestuarianus* infection in Pacific oysters (Travers *et al*., 2015). McCleary & Henshilwood (2015), provided the first evidence of *V. aestuarianus* pathology in Irish *C. gigas*, the authors noted haemocyte accumulation in the sinus and connective tissues. Here, we add to this evidence with similar findings and with additional new evidence for bacterialike clusters and widely dispersed bacteria-like cells, found in 'high' *V. aestuarianus* infected oyster seed. Infectious *Vibrio* spp. Cells often form clusters or colonies but can also be widely dispersed in late-stage infections (Bower, 1994) although this has not been shown conclusively in *C. gigas*, similar findings were observed and imaged in Kett *et al.* (2022). Indeed, the behaviour of *V. aestuarianus* in host tissues is poorly understood and this warrants further investigation (Travers *et al*., 2015).

### 4.5.4 Host-pathogen interactions

In this study, UV-B was found to negatively affect both host and parasite species. The role of UV-B radiation in host-parasite dynamics is complex. Increasing levels of UV-B could slow the transmission of parasites by inactivating free-living water-borne life-stages (Marcogliese, 2008). Conversely, UV-induced immunosuppression of hosts could lead to increased infectivity and transmission of disease (Patz *et al*., 1996, Marcogliese, 2001). However, there is also evidence that increased UV-B may enhance the immune system of animals either directly though Vitamin D production or

indirectly through improved diet as algae acclimate to UV-B exposure increasing carotenoid and mycosporine-like amino acid (MAA) production (Moeller *et al.,* 2005, Tian & Yu, 2009). Other studies have shown UV-B radiation to impact negatively on the host but not affect the pathogen (Studer *et al*., 2012) or conversely to reduce the infectivity of the parasite without altering host susceptibility (Overholt *et al*., 2012). Furthermore, Kiesecker and Blaustein, (1995) found UV-B and parasite infection to have a synergistic effect on mortality of host embryos. It seems that host-parasite relationships are affected by multiple compounding environmental conditions which alter host immune function and parasitism both independently and combined (Marcogliese, 2008, Harvell *et al*., 2009, Burge *et al*., 2014). This paper shows that UV-B radiation causes substantial mortality in oysters. This implies that the oyster immune system is likely to be similarly negatively affected. Nevertheless, this study also shows a UV-mediated transient reduction in the prevalence of a key pathogen in oysters. These data can be interpreted by similar, and parallel, deleterious effects of UV-B radiation on host and pathogen. Future work will need to explore whether these two impacts of UV-B radiation can be separated, for example by analysing UVdose dependencies and or wavelength dependencies and by reducing the exposure of younger cohorts to UV-B at culture sites. If it is possible to separate UV effects on mortality, from those on bacterial prevalence, then this would have substantial applications for the oyster industry.

## 4.6 Conclusion

UV-B radiation is a significant environmental factor in the health and survival of commercially important bivalve species and in the performance of waterborne pathogens. In this study, *C. gigas* survival decreased under UV-B exposure, especially in younger oysters with thinner shells particularly when they were out of water. UV-B radiation also reduced *V. aestuarianus* prevalence in oyster tissues. Future research would benefit from examining shell optical characteristics such as transparency and reflection. Other recommended areas of investigation include the varying impacts of different UV wavelengths and the use of in situ experiments to examine natural solar radiation and interactions with other environmental and geophysical conditions. UV as an ecosystem regulator, genotoxic stressor, and natural disinfectant should be considered important for investigations of disease outbreaks in bivalve aquaculture, which are becoming increasingly problematic in a rapidly changing marine environment.

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## 4.8 Author contributions

G.F.K: Conceptualization, methodology, data curation, visualisation, formal analysis, investigation, writing – original draft, writing – reviewing and

editing. M.J: Conceptualization, validation, writing – reviewing and editing. S.C: Funding acquisition, Conceptualization, writing – reviewing and editing, supervision. S.L: Funding acquisition, Conceptualization, methodology, writing – reviewing and editing, supervision, validation.

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# Chapter 5. Investigating the effects of solar UV radiation on host performance and pathogen development using the Pacific oyster *Crassostrea gigas* and the intertidal environment as a model.

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Keywords: UV radiation, Pacific oyster, aquaculture, shore height, pathogen partitioning

## 5.1 Abstract

Pacific oysters *Crassostrea gigas* are prone to disease outbreaks associated with pathogens (ostreid herpesvirus-1 microVar and bacteria of the genus *Vibrio*), which can decimate stocks. Such outbreaks are predicted to increase

with global warming, as *C. gigas* are pushed to the limit of their thermal tolerance and both pathogens have been shown to proliferate at higher temperatures. The holding height of *C. gigas* on the intertidal, and the resulting duration of immersion and emersion periods, exposes *C. gigas* to various biotic and abiotic factors e.g. nutrients, pathogens, temperature, desiccation and UV radiation. Very little is known about the impact of UV exposure on oyster performance and pathogen development at culture sites. In this study, a 5-month field trial to cover the summer season was carried out at two shore heights (450 m horizontal, 2 m vertical differential) typical of where oysters are held during culture to investigate *C. gigas* (spat) performance, and pathogen development. UV exposure (Met Eireann), tidal height (Marine Institute) and air temperature (Met Eireann) were noted during the trial. A total of 1000 oyster spat were split between two shore heights (HS and LS) and were sampled weekly. Mortality counts were carried out and pathogen screening for *V. aestuarianus* and OSHV1-□var was carried out by PCR and qPCR on 570 *C. gigas* gill tissues. Results indicate that higher oyster mortality was positively associated with emersion duration, including UV exposure (>2.4 kJ/m$^2$) and high air temperatures (>21 ºC). Pathogen partitioning was observed throughout the trial i.e. a higher prevalence of OsHV-1 □Var in high shore *C. gigas* while a higher prevalence of *V. aestuarianus* was detected in low shore *C. gigas*, which also had a higher infection intensity compared to high shore groups. Although both pathogens were detected throughout the trial, oyster mortality remained low at both shore heights (<10%), which would indicate that environmental conditions rather that the pathogens themselves had a greater impact on spat survival, in

particular at the start of the trial. This study shows that oyster spat mortality and pathogen infection levels are influenced by the height on the shore above Chart Datum on which the oyster are grown, and the resulting emersion time and environmental conditions. Data are discussed in relation to supporting and contrasting evidence from the literature and to commercial husbandry applications. These findings are especially relevant to aquaculture sites experiencing summer mass mortality episodes and outbreaks of *Vibrio* and/or herpesvirus related diseases.

## 5.2 Introduction

The drivers of Pacific oyster *Crassostrea gigas* mass mortality events are thought to be multi-factorial including compounding biotic factors; pathogens, physiological condition and reproductive state (Soletchnik *et al*., 2007, Cotter *et al*., 2010, Huvet *et al*., 2010) and abiotic factors; salinity, temperature, nutrient levels (Malham *et al*., 2009, Barbosa Solomieu *et al*., 2015, Prado-Alvarez *et al*., 2016 Ashton *et al*., 2020), and emersion time (Azéma *et al*., 2017, Pernet *et al*., 2019, Bordignon *et al*., 2020). The primary pathogens associated with *C. gigas* culture are bacteria belonging to the *Vibrio* genus including *Vibrio splendidus* and *Vibrio aestuarianus* and viruses such as ostreid herpesvirus-1 (OsHV-1) and variants (OsHV-1 µVar). In Ireland, *C. gigas* mass mortalities have been associated with OsHV-1 µVar, *V. aestuarianus*, warmer seawater temperatures (≥16 ºC), and sharp decreases in water salinity (Cotter *et al*., 2010, Lynch *et al*., 2012, [EFSA] 2015, PradoAlvarez *et al*., 2016 Ashton *et al*., 2020).

Globally, *C. gigas* are primarily farmed in the intertidal zone (FAO, 2018).

The most common method of oyster cultivation in Ireland is the off-bottom 'bag and trestle' method along the intertidal shore (BIM, 2020). This environment is a challenging habitat for aquatic organisms. Diurnal tidal fluctuations expose animals to widely different conditions, both biotic and abiotic including exposure to higher aerial temperatures, potential desiccation, predation, and UV radiation (Tomanek & Helmuth, 2002). Higher mortalities in older *C. gigas* that are emersed (out of water) for 4 hr have been observed in Australia (Evans *et al*., 2019). It is hypothesized that infected oysters, have a reduced ability to fully close their shells resulting in increased desiccation which impacts their fitness and survival (Paul-Pont *et al*., 2013, Whittington *et al*., 2015, Evans *et al*., 2019). However, other studies have observed that longer emersion time can reduce *C. gigas* mortality in Ireland (Peeler *et al*., 2012, Ashton *et al*., 2020), France (Azéma *et al*., 2017, Pernet *et al*., 2019), Italy (Bordignon *et al*., 2020), and Australia (Paul-Pont *et al*., 2013, Whittington *et al*., 2015) by limiting the exposure of oysters to water-borne pathogens, particularly OsHV-1 and variants. These seemingly contradictory perspectives emphasise how confounding factors determine *C. gigas* mass mortality events and highlight the importance of investigating all potential drivers.

UV radiation (UVR) can have wide-ranging effects on aquatic organisms including a genotoxic impact whereby DNA and other macromolecules are damaged resulting in deleterious effects on cells, individuals, and populations (Hader *et al*., 2015, Andrady *et al*., 2017, Williamson *et al*., 2019). Under high doses and shorter wavelengths e.g. UV-C and UV-B, UVR can damage physiological health, immune functioning, and overall fitness (McKenzie *et*

*al*., 2007, Hader *et al*., 2007). However, UVR can also have beneficial effects such as supporting immune system function with the activation of vitamin D biosynthesis pathways and DNA repair mechanisms (Rastogi & Sinha, 2015).

At the ecosystem level, UVR can impact host-pathogen systems in additive, synergistic, or antagonistic ways as shown in various aquatic systems e.g. amphibian host and fungal pathogen (Kiesecker & Blaustein, 1995), zooplankton host and fungal pathogen (Overholt *et al*., 2012), or bivalve host and bacterial pathogen (Kett *et al*., 2022). For a more extensive review of UV effects on host pathogen dynamics see Kett *et al*. (2020). UVR is a disinfectant of surface water pathogens in aquatic systems (Hader *et al*., 2015, Williamson *et al*., 2017, Bais *et al.*, 2018, Kett *et al*., 2022). Cole *et al*. (2015) found that oysters grown in surface-floating suspension trays were up to 57% less infected with pathogenic *Vibrio parahaemolyticus* compared to identical oysters grown in on-bottom trays at 5 m depth. In that study, *V. parahaemolyticus* concentrations were correlated with temperature, salinity and with depth, so that shallower waters had less *V. parahaemolyticus.* Shallower waters also have higher exposure to UVR (Tedetti & Sempéré, 2006, Smyth, 2011).

The impacts of UVR on bivalve health and pathogen development are understudied and many knowledge gaps persist (Kett *et al*., 2020). However, preliminary laboratory-based simulations have demonstrated that artificial UV-B can negatively impact *C. gigas* seed survival and reduce *V. aestuarianus* prevalence (Kett *et al*., 2022). In that controlled setting, manipulation of the abiotic factor under investigation i.e. UV-B radiation was possible in the absence of confounding effects. Conducting a similar UV exposure study under outdoor conditions, at a *C. gigas* culture site generates

multiple challenges, given the fluctuating nature of the intertidal zone and the multifactorial nature of *C. gigas* mortalities.

The focus of this study was firstly to assess existing literature surrounding the impact of oyster cultivation height and/or emersion time on host and pathogen performance. Secondly, a field experiment was conducted which took both a "host-centric" and "pathogen-centric" approach to investigate how UV-B exposure may influence host performance whilst determining its effect/s on pathogen proliferation and development using the *C. gigas* : *V. aestuarianus* : OsHV-1 µVar intertidal environment model. The use of disease resistant, selectively bred *C. gigas* reduced the role that the pathogens had on oyster performance while the two shore holding heights were chosen to have oysters emersed for variable lengths of time. This experimental design facilitated a comparison between cohorts that had more time (+4 hrs) out of water with resulting higher exposure to UVR (high shore (HS) cohort) and air temperatures and those that had a lower exposure to UVR and air temperatures (low shore (LS) cohort). Additional environmental variables that determine exposure to UVR, such as corresponding tidal height, were also examined. Findings from this study will build on existing knowledge of the role that localised environmental parameters play on the performance of selectively bred *C. gigas* and pathogen prevalence but more importantly it will shed light on the effects of UV exposure on a global intertidal cultured species and significant pathogen groups.

## 5.3 Methods

### 5.3.1 Field site, *C. gigas* spat and experimental design

The field trial was carried out at a commercial *C. gigas* culture site in Dungarvan Bay (N: 52.06431, W: -7.59412), Ireland (Figure 1), where *V. aestuarianus* and OsHV-1 µVar are endemic. Tidal height in Dungarvan Bay typically ranges from – 0.26 m to + 5 m from Chart Datum (Met Éireann tidal observations). The trial ran from July to October 2018. This sampling time period was chosen as oyster mortalities typically occur during these months at Dungarvan Bay. Average monthly (daily) hours of sunshine range from 170 (6.8) sunhours (July), 160 (6.2) sunhours (August), 120 (5) sunhours (September) to 90 (4.4) sunhours (October) (weather-and-climate.com, en.climate-data.org). The average monthly air temperature ranges from 16 ºC (July), 15 º C (August), 14 º C (September) to 12 º C (October) (en.climatedata.org).

Six bags of *C. gigas* spat (n = 1000 per bag, mean whole wet weight = 0.12 g, mean length = 12.5 mm, age = <3 months) were relayed at two different shore heights (three bags per location) in the intertidal zone (Figure 1). The spat used in this trial were reared in a nursery at Dungarvan for 4 to 6 weeks after they were imported from France, where they were bred to be resistant to ostreid herpesvirus OsHV-1 µVar (Dégremont *et al*., 2015). The spat were exposed to untreated seawater from the bay, ambient temperatures and were not exposed to UVR while in the nursery.

The *C. gigas* were subsequently held in PVC mesh bags placed on trestles at two shore heights that were 450m apart along the cross-shore gradient (Figure

1). Seed located on the “High Shore” (HS) were held at 1 meter below sea level at high tide while seed on the “Low Shore” (LS) were held at 3 meters below sea level. This resulted in HS oysters being emersed from the tide for an average of 4 additional hours compared to the LS oysters between the tidal flood (incoming/rising tide) and ebb (outgoing/falling tide) (www.tideforecast.com, Dungarvan Shellfish oyster producers pers. comm.).

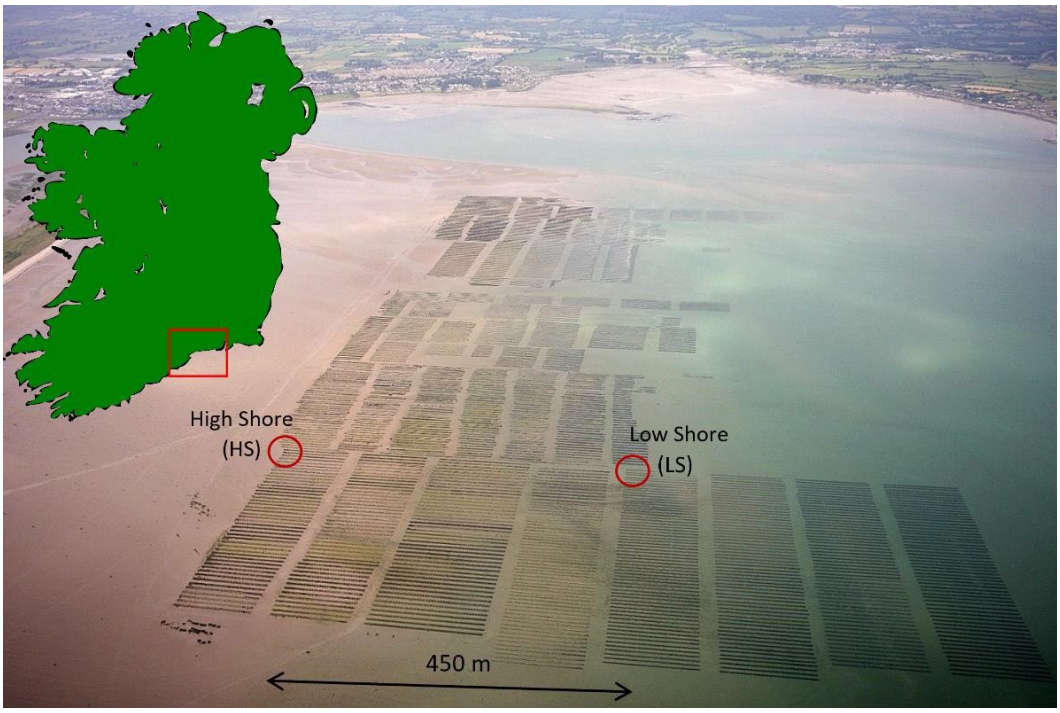



Figure 1. Aerial view of oyster trellis and bag structures in Dungarvan bay, showing High Shore (HS) and Low Shore (LS) sampling points circled in red.

Photo: Ash Bennison, ObSERVE.

### *5.3.2 C. gigas* sample regime and processing

An initial sample of *C. gigas* (n = 30) was taken for morphometrics (determination whole wet weight using a balance scales and length using a vernier callipers) and pathogen screening before oyster bags were relayed.

Samples (n = 30/shore height i.e. 10 seed per bag (x 3) per shore height (x 2) = total of 60 oysters/sample time) were taken weekly during July and August (n = 7 sample points from weeks 1 to 8) and monthly in September and October (n = 2 sample points in weeks 12 and 16). An overall total of 540 seed (n = 270/shore height) were sampled from the site during the experiment.

Mortality (%) was counted in the field at each sample time point (weekly in July and August, monthly in September and October) by removing 100 oysters at random from each of the three replicate bags and counting the number of empty shells or moribund oysters which did not respond to external stimuli i.e. tapping on open shells. Unresponsive individuals were counted as moribund and responsive individuals were returned to their respective bags. This method gave three figures for percentage mortality, which were averaged for each shore height.

Gill and mantle tissue was removed for the molecular screening of each pathogen and were stored at -20 ºC.

### 5.3.3 Diagnostic methods

#### *5.3.3.1 Environmental data*

Air temperature (meteorological) and UVR data were recorded using data from the Irish national meteorological organisation, Met Éireann (https://www.met.ie/). UV-B readings were attained from the Met Éireann observatory in Valentia Island, Kerry, located at the same latitude as the Dungarvan field site (52.0 º N) but 2.3º further to the west, equivalent to a distance of ~180 km. Readings integrate UV-intensities between 285 nm and 325 nm. These data were the most accurate UV-B records which could be

obtained due to the high specification Brewer #088 spectrophotometer used at the Met Éireann site. Daily UV indices, measured at both Dungarvan and Valentia were used to validate the data.

#### 5.3.3.2 Molecular methods

*a) DNA extraction*

Gill tissue samples were placed into 100 µl of 10% chelex solution and positioned into a Hybaid thermocycler at 99 ° C for 1 hr 10 mins (Walsh *et al*., 1991, Lynch *et al*., 2012). Extracted genomic DNA samples were stored at -20 ° C. To ensure the quality and quantity of DNA extracted was of optimal values for qPCR and novel PCR, a NanoDrop 100 spectrophotometer was used to check the OD 260/280 and concentration of genomic DNA in samples, as per the manufacturers protocol (Maniatis *et al*., 1982).

*b) Quantitative polymerase chain reaction (qPCR) for Vibrio aestuarianus*

Quantitative PCR (qPCR) was carried out to determine if *V. aestuarianus* DNA was detected in oyster groups and if so, at what intensity (McCleary & Hensilwood, 2015). The primers used in this qPCR mix (*dnaJ* f420, *dnaJ* R456) are specific to *V. aestuarianus*. Oysters were classified into four infection intensity groups based on their Ct value with a Ct of >37 being classified as 'Uninfected' (as per McCleary & Hensilwood, 2015), Ct 35 – 37 as 'Low infection', Ct 30 – 35 as 'Medium infection' and Ct <30 as 'High infection'. Negative controls (dd$H_2O$) and positive controls (5 µl of purified *V. aestuarianus* genomic DNA (Marine Institute, Ireland)) were included in each qPCR run.

*c) Polymerase chain reaction (PCR) for ostreid herpesvirus OsHV-1 µVar*

The presence or absence of ostreid herpesvirus-1 µVar was confirmed using the thermocycling conditions and primer pairs (OHVC/OHVD) (Lynch *et al*., 2013). Negative controls (dd$H_2O$) and positive controls (2.5 µl of purified OsHV-1 µVar genomic DNA (Ifremer, EU reference lab for mollusc disease) were included in each PCR run.

Visualisation of PCR products using SYBR safe dye was carried out by gel electrophoresis on a 2% agarose gel which was ran at 110 V for 60 mins before being illuminated by UV light. Expected amplicons of 385 bp confirmed the presence or absence of OsHV-1 µVar.

#### *5.3.3.4 Statistical analysis*

Statistical analyses were carried out using IBM SPSS version 29.0. Overall oyster mortality was anlaysed using a Chi square goodness of fit test while a linear regression was used to analyse the effect of shore height, UV-B and air temperature on oyster mortality over the sampling points of the experiment. A univariate general linear model was used to analyse pathogen infection prevalence under high and low UV periods with sampling period set as a random factor and shore height as a covariate.

## 5.4 Results

Table 1 highlights the literature found exploring the effect of shore height and/or emersion time on oyster mortality in the presence of common pathogens. Three examples were found in which oyster mortality increased with increased shore height and/or emersion time (Trimble *et al*. 2009, Evans *et al*. 2019, Bodenstein *et al*. 2021). The most likely explanations for this as concluded by the authors were the physiological stressors associated with

emersion conditions, namely extreme air temperatures and desiccation. This is particularly relevant for OsHV-1 infected adult oysters, as the pathological symptom of valve gaping exposes tissues to the aerial environment (Llabres *et al*., 2013, Evans *et al*., 2019), and may cause oyster desiccation. UVR was not investigated as an environmental variable of aerial exposure in these studies despite the extensive research regarding UVR as a stressor and/or environmental regulator in other animal and plant groups of the intertidal and shallow aquatic habitats (Kunze *et al*., 2021). However, eight studies were found demonstrating a contrasting result, in which oyster mortality decreased when held at increased shore height or emersion time (Soletchnik *et al*. 2007, Peeler *et al*. 2012, Paul-Pont *et al*. 2013, Whittington *et al*. 2015, Azema *et al*. 2017, Bookelaar, 2018, Pernet *et al*. 2019 Ashton *et al*., 2020). This is primarily thought to be because of the protective effect of increased emersion which reduces the exposure time of oysters to water-borne pathogens. Another explanation offered for this observation is reduced metabolic and growth rates of oysters held on the high shore, due to reduced food availability. This decrease in metabolic activity may have led to reduced viral replication and transmission (EFSA, 2015).

**Table 1. Studies invetigating the impact of shore height and/or emersion time on oyster and pathogen performance.**

| Host species | Pathogen | Host age | Expt. Design | Height | Time | Environmental | Key finding | Potential explanation | Location | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|
| ***Crassostrea gigas*** | OsHV-1 | Spat and adult | Lab trial to test tidal emersion on OsHV-1 infection Constant vs twice daily emersion | n/a | 10 hr emersion vs 0 hr emersion | Emersion only | Mortality increased 4x under tidal (emersion) conditions | Infected adult oysters held at high shore as valve gaping exposed animals to environmental stressors | Australia | Evans *et al*. 2019 |
| ***Crassostrea virginica*** | n/a | 12month juvenile | Oysters exposed to 18, 24, or 48h desiccation and tumbling | n/a | 18-, 24-, or 48-hours emersion | n/a | Mortality increased in high stress 48 hr desiccation groups | Mortality linked to physiological stress through desiccation. Environmental effects may have contributed but not confirmed | USA | Bodenstein *et al*. 2021 |
| ***Ostrea lurida*** | n/a | Spat | Investigating factors inhibiting population recovery | n/a | 8% greater emersion time | Emersion only | Mortality increased by 80% in emersion treatment | Mortality linked to physiological stress e.g. air temperatures and other environmental factors | USA | Trimble *et al*. 2009 |
| ***Crassostrea gigas*** | OsHV-1 | All stages | Mortality monitoring over 3 year period at commercial oyster site | n/a | n/a | Emersion, temperature, salinity | Mortality decreased with simulated emersion conditions | Mortality linked to changes in environment e.g. water temps >20 $^{o}$C and salinity <20. Protective effect of emersion. | Ireland | Ashton *et al*. 2020 |

**Table 1. Studies investigating the impact of shore height and/or emersion time on oyster and pathogen performance. (cont.)**

| Host species | Pathogen | Host age | Expt. Design | Height | Time | Environmental | Key finding | Potential explanation | Location | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|
| ***Crassostrea gigas*** | OsHV-1 | Spat, juvenile, adult | Farmer survey data to investigate mortality events | n/a | < 8hr vs >8 hr emersion | Emersion only | Mortality decreased in groups emersed less than < 8 hrs | Emersion and low growth rate may mean less disease susceptibility and pathogen exposure. | Ireland | Peeler *et al*. 2012 |
| ***Crassostrea gigas*** | OsHV-1 | Spat | Farmer monitoring of sites around France | 2 m | n/a | Temperature | Mortality decreased in oysters held higher | Protective effect of emersion reducing exposure to OsHV-1 | France | Pernet *et al*. 2019 |
| ***Crassostrea gigas*** | OsHV-1 | Spat and adult | Modified inter- and sub-tidal growing bags | 300 mm | Reduced immersion | Temperature | Mortality decreased in adult oyster held at height | Protective effect of emersion reducing exposure to OsHV-1 | Australia | Whittington *et al*. 2015 |
| ***Crassostrea gigas*** | OsHV-1 | Spat and adult | Modified growing trellis 300 mm higher than control | 300 mm | Reduced immersion | Temperature, salinity | Mortality decreased in adult oyster held at height | Protective effect of emersion reducing exposure to OsHV-1 | Australia | Paul-Pont *et al*. 2013 |
| ***Crassostrea gigas*** | OsHV-1 and Vibrio | Seed and juvenile | Two shore heights used to investigate emersion on mortality and health of native and disease resistant groups | n/a | n/a | Shore height (emersion) | Mortality decreased in high shore groups with increased emersion | Protective effect of emersion reducing pathogen exposure for juveniles.<br>Seed oysters more susceptible to environmental stressors of emersion | Ireland | Bookelaar, 2018 |

**Table 1. Studies investigating the impact of shore height and/or emersion time on oyster and pathogen performance. (cont.)**

| Host species | Pathogen | Host age | Expt. Design | Height | Time | Environmental | Key finding | Potential explanation | Location | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|
| ***Crassostrea gigas*** | OsHV-1 | Spat | Multiple field experiments investigating husbandry practices with mortality | n/a | n/a | Hydrodynamics and habitat type | Spat survival increased with raised growing height | Protective effect of emersion reducing pathogen exposure | France | Soletchnik *et al*. 2011 |
| ***Crassostrea gigas*** | OsHV-1 | Spat | Field experiments monitoring OsHV-1 infection and mortality between oyster families, pathogen resistance and shore height | Three growing heights (high, medium, low) | Heights corresponded to emersion time of 25%, 12% and 2% [of day] | Growing height and pathogen resistant vs naive | Mortality decreased in high shore groups. Highest mortality in low shore after OsHV-1 outbreak. Resistance to OsHV-1 affected outcome | Protective effect of emersion reducing pathogen exposure | France | Azema *et al*. 2017 |

1

### 5.4.1 Environmental results

*UV exposure periods – Solar noon and low tide*

Solar noon is when the sun reaches the highest point in the sky for the area of interest. At Dungarvan the period around solar noon is between 10 am and 3 pm (Figure 1, supplementary material). Figure 2 depicts the tidal range throughout the month of July 2018 i.e., 'Week 1' – 'Week 4' of the field trial period. Tidal range did not differ significantly throughout the remainder of the trial. In figure 2 below, and in figures 4 – 7 following, yellow bands show periods of "high UV" exposure in which the period around solar noon coincides with low tide. Thus, these are episodes when the spat at Dungarvan were emersed (out of water) when solar UVR was at its highest intensity and animals were directly exposed to UVR without the protective effect of water which attenuates and scatters solar radiation. Conversely, grey bands show periods of "low UV" exposure, i.e. when the tide was high (>3.5 m) in the period around solar noon, thus offering a protective shielding from seawaters optical properties

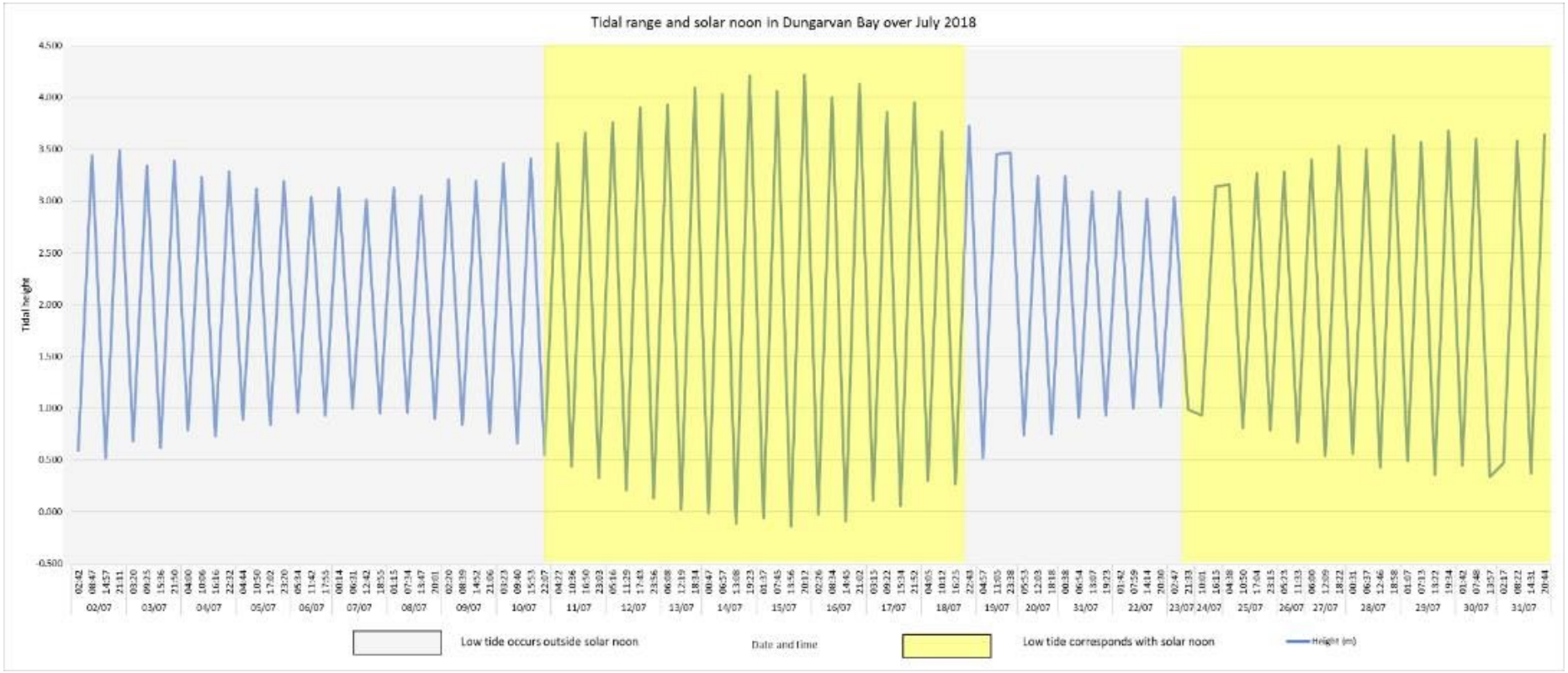



Figure 2. Tidal range in July 2018 showing when the period around solar noon corresponded with low tide (yellow) or when low tide occurred outside solar noon (grey). This period around solar noon is defined as the 5 hr window between 10 am to 3 pm.

### 5.4.2 Oyster mortality

Mortality was significantly higher in the HS cohort (20%, n = 52/270) compared to the LS cohort (12%, n = 32/270) ($\square^{\square}$ = □□□□□ df = 1, p = 0.017). HS mortality comprised 61% of total while LS mortality accounted for 39% of total (Figure 3). Mortality increased in both groups during July when mean UV-B levels were >2.4 kJ/m$^2$/day, air temp. was >21 ºC, and solar noon period corresponded with low tide (Figure 4).

In periods of high UVR (solar noon period corresponding with low tide) during summer months (July – August when average daily UV irradiance was high, i.e.>1.5 kJ/m$^2$), cumulative oyster mortality was consistently higher in the HS cohort compared to the LS cohort (Figure 4). Conversely, in periods of low UVR and during the autumn months (Sept. – October), cumulative mortality increased more in the LS cohort (Figure 2, 4). Linear regression analysis shows that oyster mortality was positively associated with both UV-B and air temperature (F = 3.66, df = 2, p = 0.05). Oyster mortality was more strongly correlated to UV-B ($R^2$ = 0.46, p = 0.027) than air temperature ($R^2$ = 0.24, p = 0.14). This observation is demonstrated in the figure 4 where the HS:LS mortality ratio is displayed. A ratio value of 1 denotes that mortality levels are equal while any value >1 shows higher mortality in HS groups than LS groups at that time.

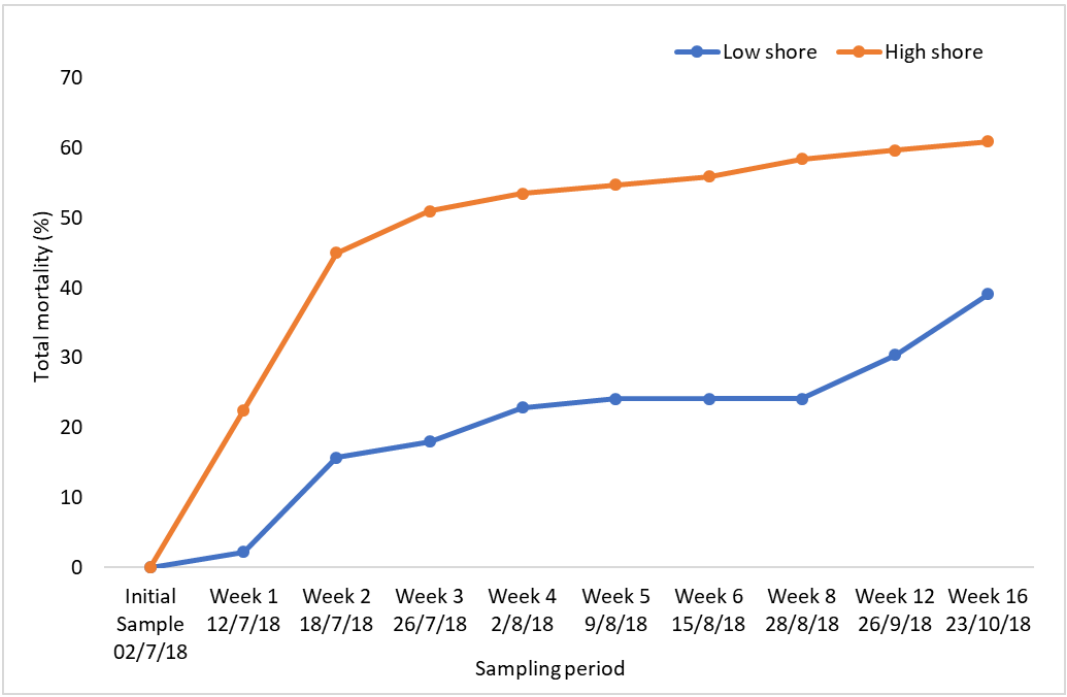
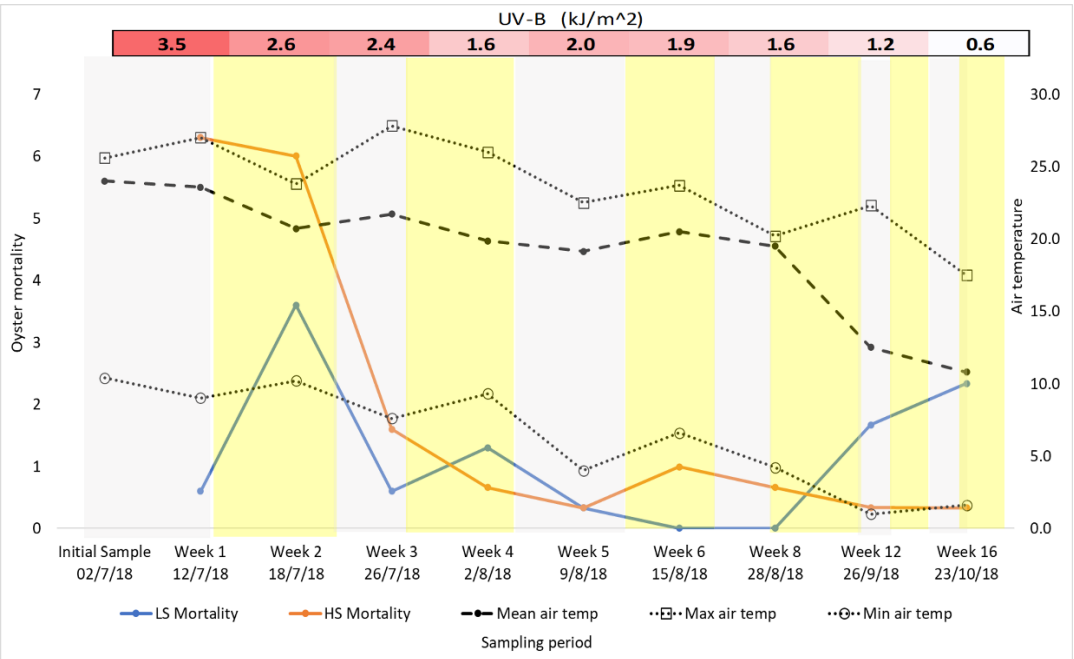



Figure 3. Cumulative mortality of oyster groups in 'low shore' (blue) and 'high shore' (orange) as % of overall mortality.

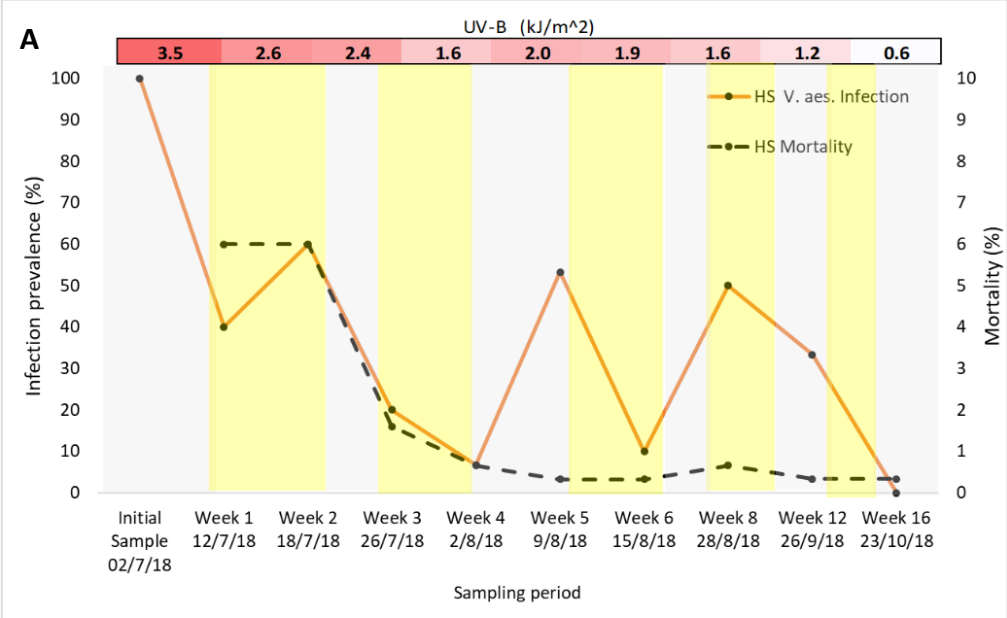


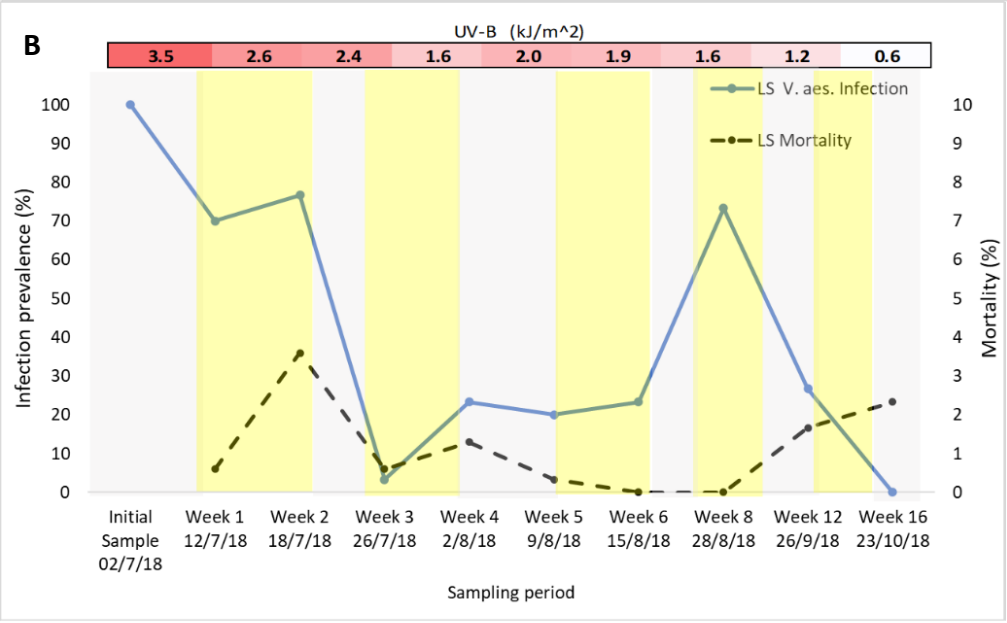

| | Initial Sample 02/7/18 | Week 1 12/7/18 | Week 2 18/7/18 | Week 3 26/7/18 | Week 4 2/8/18 | Week 5 9/8/18 | Week 6 15/8/18 | Week 8 28/8/18 | Week 12 26/9/18 | Week 16 23/10/18 |
|---|---|---|---|---|---|---|---|---|---|---|
| HS:LS ratio | - | 10.00 | 1.67 | 2.67 | 0.51 | 1.00 | 1.33 | 1.66 | 0.20 | 0.14 |
| UV-B (kJ/m2) | 3.5 | 3.49 | 2.62 | 2.44 | 1.64 | 2.04 | 1.90 | 1.60 | 1.18 | 0.63 |
| Mean air temp. (°C) | 24.0 | 23.6 | 20.7 | 21.7 | 19.9 | 19.1 | 20.5 | 19.5 | 12.5 | 10.8 |

Figure 4. Oyster mortality (average % mortality of 100 oysters randomly selected) of oyster groups in 'low shore' (blue) and 'high shore' (orange). Broken black line denotes daily mean air temperatures (temp.) over the 7 day period preceding each sampling period with min. (circular markers) and max. (square markers) values represented by dotted black line. Average UV-B ($kJ/m^2$) for the 7 day period preceeding each sampling period is shown in the horizontal bar along the top. Yellow vertical bars denote "high UV" exposure periods when the solar noon peiod corresponds with low tide. Grey bars indicate "low UV" exposures when the solar noon period occurred when oysters were submerged under the tide. The table underneath shows oyster mortality ratio between Low Shore (LS) and High Shore (HS) groups and the corresponding UV-B and air temperature.

### 5.4.3 *Vibrio aestuarianus* detection in *C. gigas* spat

A total of 570 individuals were screened for *V. aestuarianus* throughout the trial, 270 from the HS and the LS, and 30 from the 'Initial sample'. In the initial *C. gigas* sample (2nd July), *V. aestuarianus* was detected in 100% of the samples while at the end of the trial (Week 16; 23rd October), *V. aestuarianus* was not detected in either shore cohort (Figure 5). Overall, *V. aestuarianus* was detected in 35.2% (n = 95/270) of *C. gigas* sampled from the LS cohort, and in 30.4% (n = 82/270) of *C. gigas* sampled from the HS cohort ($\chi^2 = 1.42$, df = 1, $p > 0.05$). Pathogen partitioning was evident in the intensity of infections as detected *via* qPCR, comparing the numbers of individuals in each infection level group. More individuals with a "high" degree (CT: <30) of *V. aestuarianus* infection were observed in LS groups (6.7% (18/270)) compared to HS groups (0.4% (1/270) ($\chi^2 = 15.76$, df = 1, $p < 0.001$)) while "low" and "medium" intensities (CT: 35 – 37 and 30 – 35, respectively) were similar in both shore cohorts (HS - 17.8% (48/270) and 12.2% (33/270) respectively; LS -17% (46/270) and 11.5% (31/270) respectively). When oysters were sampled during or after periods of high UV exposure (low tide at solar noon, yellow bands), *V. aestuarianus* detection was consistently lower in HS *C. gigas* compared to LS *C. gigas* (figure 5 – weeks: 1, 2, 4, 6, *8*). Conversely, when oysters were sampled during periods of low UV exposure (grey bands), a higher detection of *V. aestuarianus* was observed in HS *C. gigas* compared to LS *C. gigas* (figure 5 – weeks: 3, 5, 12). *V. aes.* infection detection and mortality in the HS and LS cohorts is shown in Figure 5.




**C**

| | Initial Sample | Week 1 | Week 2 | Week 3 | Week 4 | Week 5 | Week 6 | Week 8 | Week 12 | Week 16 |
|---|---|---|---|---|---|---|---|---|---|---|
| LS V. aes. Infection | 100 | 70 | 76.7 | 3.3 | 23.3 | 20 | 23.3 | 73.3 | 26.7 | 0 |
| HS V. aes. Infection | 100 | 40 | 60 | 20 | 6.7 | 53.3 | 10 | 50 | 33.3 | 0 |
| HS:LS Infection Ratio | 1 | 0.57 | 0.78 | 6.06 | 0.29 | 2.67 | 0.43 | 0.68 | 1.25 | 0 |

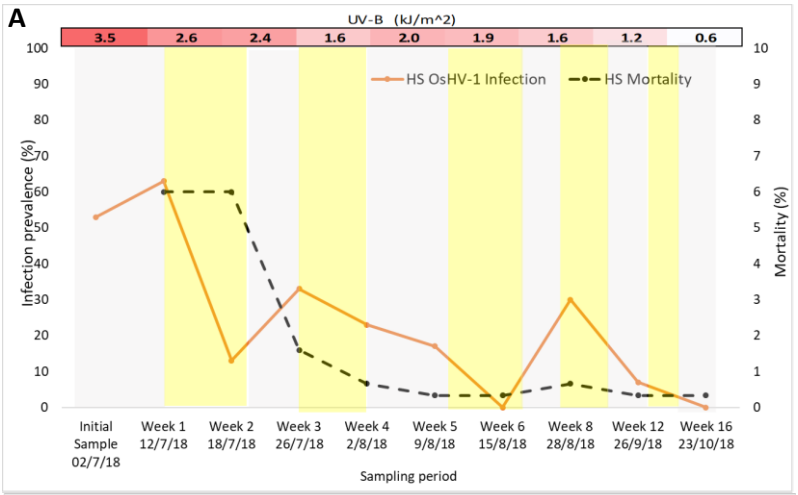
Figure 5. Detection of *Vibrio aestuarianus* (*V. aes.*) in **A**: High shore (HS) and **B**: Low shore (LS) with mortality (%) of the same groups in broken black lines. **C:** shows the ratio of HS/LS detection whereby HS:LS = 1 indicates no difference between treatments and HS:LS = 0.5 or 2 indicates that HS detection was half or twice that of LS. Overall average UV-B (kJ/m$^2$) in horizontal scale

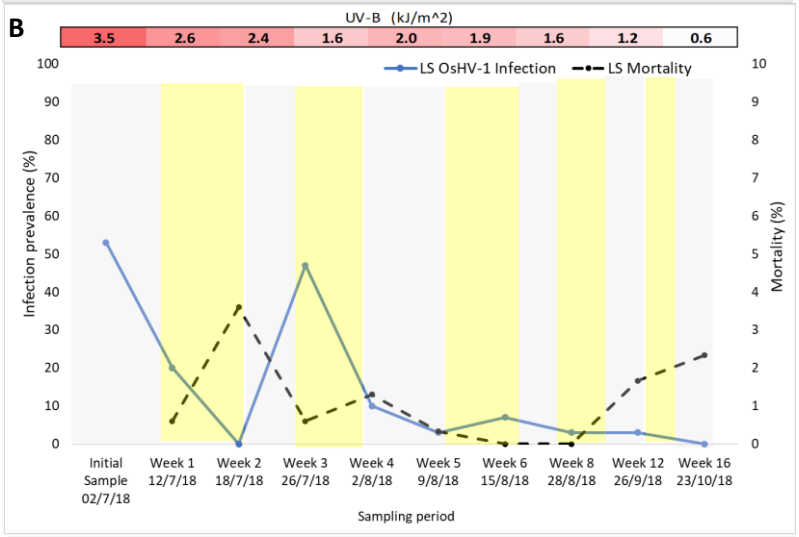
bar and periods of high exposure i.e. solar noon corresponds with low tide denoted in yellow columns and periods of low UV exposure in grey columns. N = 30 oysters/ treatment/ sample point. HS:LS infection ratio of 1 shows equal detection of *V. aestuarianus,* values <1 show that HS oysters had lower pathogen detection and values <1 show higher pathogen detection in HS oysters.

### 5.4.4 Ostreid herpesvirus-1 microvar (OsHV-1µVar) detection in *C. gigas* spat.

A total of 570 *C. gigas* were screened for OsHV-1 µVar (n = 270 HS, n = 270 LS, n = 30 'Initial sample). In the initial sample, OsHV-1 µVar was detected in 53.3% *C. gigas* and continued to be detected throughout the summer months (July – September) (figure 6). The virus was not detected in the October samples. HS *C. gigas* had a higher (20.7% (n = 56/270) detection, compared to the LS *C. gigas* (10.4% (n = 28/270) ($\square^2$ = 11.05, df = 1, p < 0.001)). The highest detection of OsHV-1 µVar was observed in HS *C. gigas* in Week 1 (63.3%, n = 19/30) while highest OsHV-1 µVar detection in LS *C. gigas* was in Week 3 (46.7%, n = 14/30).

When oysters were sampled in, or following, periods of 'High UV' (low tide at solar noon), OsHV-1 □Var detection was higher in HS *C. gigas* compared to LS *C. gigas* (Figure 6, weeks: 1, 2, 4, 8), although this result was not found to be significant (F = 0.174, df = 1, 0 = 0.68).




C

| | Initial Sample | Week 1 | Week 2 | Week 3 | Week 4 | Week 5 | Week 6 | Week 8 | Week 12 | Week 16 |
|---|---|---|---|---|---|---|---|---|---|---|
| LS OsHV-1 Infection | 53.00 | 20.00 | 0.00 | 47.00 | 10.00 | 3.00 | 7.00 | 3.00 | 3.00 | 0.00 |
| HS OsHV-1 Infection | 53.00 | 63.00 | 13.00 | 33.00 | 23.00 | 17.00 | 0.00 | 30.00 | 7.00 | 0.00 |
| OsHV-1 HS:LS Infection ratio | 1.00 | 3.15 | -- | 0.70 | 2.30 | 5.67 | -- | 10.00 | 2.33 | -- |

Figure 6. OsHV-1 □Var detection in **A**: High shore (HS) and **B**: Low shore (LS) oyster groups alongside oyster morality of the same. **C:** Shows the ratio of HS:LS detection whereby HS:LS = 1 indicates similar infection levels of each shore height. Periods of high UV exposure in yellow vertical columns while horizontal scale bar shows average daily UV-B (kJ/m$^2$). N = 30 oysters/

7 treatment/ sample point. HS:LS infection ratio of 1 shows equal detection of

8 OsHV-1 µVar, values <1 show that HS oysters had lower pathogen detection

9 and values <1 show higher pathogen detection in HS oysters.

### 5.5.5 Co-detection of OsHV-1 µVar and *V. aestuarianus* in *C. gigas* spat

In the initial *C. gigas* sample, both OsHV-1 µVar and *V. aestuarianus* were detected simultaneously in six individual spat (20% (n= 6/30)). In total, both pathogens were detected simultaneously in 19 HS individuals and 17 LS individuals. Both pathogens were not detected simultaneously in any HS individuals on 'week 1' and in LS individuals on 'week 2'.

As the trial progressed, co-detection of both pathogens in the same individual oyster was highest in the HS spat at Week 5 (17%, n = 5/30), followed by weeks 2 and 8 (10%, n = 3/30). In the LS group, co-detection of both pathogens in the same individual oyster was lower after week 2 (<10%, min = 0/30, max = 2/30). The variation between co-infection levels was not found to vary significantly between shore heights ($\chi^2$ = 12.2, df = 8, p = 0.13).

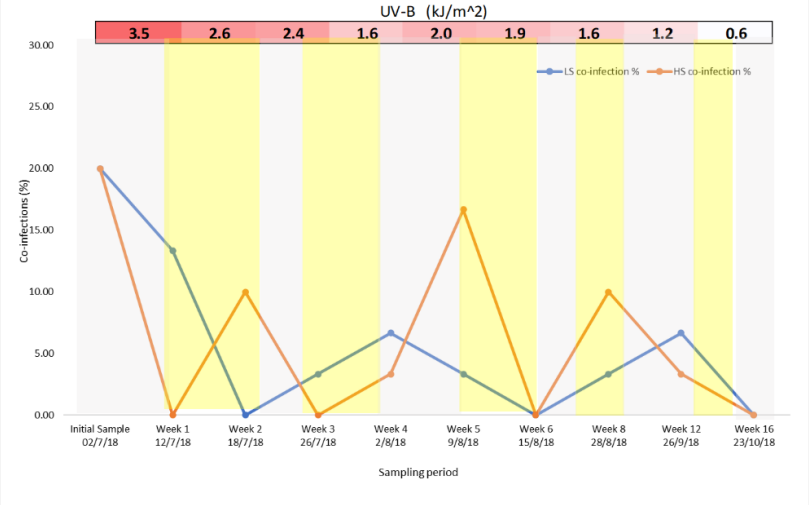


Figure 7. Simultaneous detection of OsHV-1 µVar and *Vibrio aestuarianus* in individual spat in HS groups (orange) and LS groups (blue) for the duration of the field experiment.

## 5.6 Discussion

This research shows that oyster mortality increased in oyster spat kept on the high shore which experienced increased emersion and exposure to high air temperatures and UVR, compared to low shore oyster spat which were submerged for longer time periods. Similar findings have been described in the literature and were attributed to the physiological stress of increased emersion, particularly, high air temperature (Trimble *et al*. 2009, Evans *et al*. 2019, Bodenstein *et al*. 2021). UVR is rarely considered as a factor in bivalve emersion studies or bivalve host-pathogen relationship studies (Kett *et al*., 2020). While this observation could be influenced by a range of factors, we note a strong relationship with UV-exposure. Most deaths of HS oysters occurred when low tide coincided with the 'high UV exposure' period around solar noon. This emphasises that UV exposure can be an important determinant of oyster mortality. However, it is likely that exposure to other unfavourable environmental factors e.g. high temperature, also increase when low tide coincides with solar noon. An important goal of future mortality studies will be to dissect these confounding environmental factors, and their individual and combined effect on mortality.

Remarkably, in the Autumn months (September – October 2018) when temperature and UV-B dropped below 20 $^{o}$C and 1.5 kJ/m$^2$ respectively, the greatest mortality was observed in low shore oysters which are least UV exposed. It remains to be proven whether this is a direct UV effect. Another

possible explanation for this observation is the theory that growth rates are strongly linked to immune function, and the greater exposure to food particles in LS autumn groups may have diverted some energy from immune protection to rapid growth. This phenomenon was recently noted in fast-growing, wellfed *C. gigas* challenged with OsHV-1 (Pernet *et al*., 2019) and in *Ostrea edulis* and its protozoan pathogen *Bonamia ostreae* (Egerton *et al*., 2020).

Pathogen partitioning was observed in this study in that. Oysters held on the high shore site had a higher detection of OsHV-1 µVar and lower detection of high intensity/high abundance of *V. aestuarianus* in spat compared to their low shore counterparts. Numerous studies have shown that *Vibrio* bacteria, particularly *V. aestuarianus*, are negatively impacted by exposure to UV-B radiation as observed in solar deactivation of water borne pathogens (Joux *et al*., 1999, Williamson *et al*., 2017, Kett *et al*., 2022). In addition, many herpes viruses are known to be activated by UV irradiation including human simplex herpes virus (Norval *et al*., 2007). This information would support the findings of this study in that increased UV exposure as experienced by oysters on the high shore may have played a role in the deactivation of the bacterial *V. aestuarianus* and the activation of osterid herpesvirus OsHV-1 µVar. Conversely, low shore oysters, in periods of low UVR exposure will have lacked this beneficial UVR effect.

It is important to note that both OsHV-1 µVar and *V. aestuarianus* manifest pathological symptoms in infected oysters, which include the reduced ability to close their shell properly (gaping). This will result in infected oysters' soft tissues having greater exposure to increased UVR, desiccation and predation during tidal emersion. However, in submerged conditions, this symptom

increases their exposure to secondary infections and potential additional biotic and abiotic factors (Paul-Pont *et al*., 2013, Whittington *et al*., 2015, Evans *et al*., 2019).

Investigations such as this one into the role of shore holding height in oyster and pathogen performance are highly important, especially given the varying findings observed previously in this research area. As UVR is closely associated with air temperature, cloud cover, rainfall, and ozone, and as the Earth's climate is changing and will continue to change, findings from this study will provide the shellfish sector with additional factors to consider when holding younger cohorts on the intertidal zone, especially during periods of fluctuating or extreme weather conditions such as heatwaves, and in periods of disease outbreaks.

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## 5.8 Author contributions

G.F.K: Conceptualization, methodology, fieldwork, data curation, visualisation, formal analysis, investigation, writing – original draft, writing – reviewing and editing. M.J: Conceptualization, validation, writing – reviewing and editing. S.C: Funding acquisition, Conceptualization, writing – reviewing and editing, supervision. S.L: Funding acquisition, Conceptualization, methodology, writing – reviewing and editing, supervision, validation.

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# Chapter 6: Discussion and Conclusion

## 6.1 Context

*Crassostrea gigas* is an economically important bivalve mollusc, contributing to sustainable food security and global ocean economies. Yet, the industry faces serious threats in episodic summer mortality episodes, disease outbreaks and climate impacts (FAO, 2018). Summer mortality episodes are largely driven by several, often cumulative factors, environmental; temperature, and salinity, pathogens; *V. aestuarianus* and OsHV-1 µVar, and physiological condition of the hosts (Cotter *et al*., 2010, [EFSA], 2015, Prado-Alvarez *et al*., 2016). UV radiation (UVR) is an ecosystem modulator with known biochemical pathways affecting intertidal ecology, aquatic pathogens, and animal health (Barnes *et al.*, 2022). Incident UV levels in aquatic ecosystems are likely to change with climate-related shifts in cloud cover, precipitation,

and land run-off affecting water optical properties (Bais *et al.*, 2018). Yet, the role of UVR in *C. gigas* summer mass mortality events is unclear. This was identified as a key research gap in understanding these mortality events and securing the *C. gigas* culture industry against the threats of climate change and emerging diseases. To address this a series of consecutive steps were designed which would collate and assess the existing knowledge to build an informed base upon which to formulate hypotheses, identify and construct the tools required to tackle the research challenges, and conduct lab- and field-based experiments to provide a holistic, evidence-based understanding of the *C. gigas*-pathogen-environment model (Figure 1).

The focus of this research has been on the host-pathogen-environment system using *C. gigas* as a model host species and the common pathogens associated with mortality events i.e. *V. aestuarianus* and OsHV-1 µVar, in an intertidal habitat with a particular emphasis on UVR effects. Understanding the hostpathogen-environment model of *C. gigas* is key to securing the intertidal shellfish culture sector against the threats of rapidly changing climate and disease outbreaks (EFSA, 2015). The outcomes of this research will be relevant to the intertidal bivalve aquaculture industry globally, as well as shedding further light on aquatic host-pathogen ecology. *C. gigas* is cultured worldwide and every major producing nation experiences mass mortality episodes in the summer months, often associated with these pathogens. In addition, the diagnostic tools designed in this study can be used to detect and localise *Vibrio* bacteria, not exclusive to *C. gigas*, allowing for blanket screening of *Vibrio* by PCR and for improved understanding of routes and sites of *Vibrio* infection in any histological sample. The lab-based experiment here

provides clear evidence for the negative impact of supplemental UV-B radiation on *C. gigas* survival and on *V. aestuarianus* infection prevalence and intensity. The field-based experiment shows clearly that oyster performance is affected by growing height and pathogen partitioning occurs across the intertidal gradient. These findings are important for understanding the wider host-pathogen-environment ecology as well as how specific aquaculture husbandry can affect disease susceptibility and mortality episodes.

## 6.2 Research questions and answers:

1) **What do we know about the impact of UVR on intertidal bivalve species and aquatic pathogens, can we use that information to hypothesise the effect of UVR, particularly UV-B, on *C. gigas*, *V. aestuarianus*, and Osterid herpesvirus-1 microVar (OsHV-1 μVar)?**

*Kett, G.F., Culloty, S.C., Lynch, S.A. and Jansen, M.A. (2020) "Solar UV radiation modulates animal health and pathogen prevalence in coastal habitats knowledge gaps and implications for bivalve aquaculture." Marine Ecology Progress Series, 653, pp.217-231.*

Metrics: Citations: 3. Readers: 115. Social media impacts: 6 (06/10/2022).

Literature shows that UVR has mixed effects on aquatic invertebrates and their host-pathogen dynamics. Evidence exists for synergistic, additive, and antagonistic effects between UVR and infection outcomes in various aquatic host-pathogen relationships. For bacteria, UVR frequently has a negative

impact, particularly some *Vibrio* species which demonstrate high sensitivity to UVR exposure. Encapsulated pathogens e.g. cysts, metacercaria can withstand higher UVR exposure without notable effect. Viruses show more mixed effects with UV-C sterilising some viruses in laboratory conditions, yet many appear to be unaffected or have very high tolerance to UVR exposure and some studies showing viral activation under UV exposure. Not enough data could be found on UV impacts on bivalve health.

These findings show that an important gap exists in the literature which needs to be addressed to properly understand environment–host–pathogen relationships and ensure predictability and security in the bivalve aquaculture sector. Most notably, the lack of information available on bivalve species health and performance under UVR exposure. From this review of existing literature, it can be hypothesised that UVR would negatively impact *C. gigas*, especially in the early life stages as was observed in other molluscan and crustacean species. It is also predicted that UVR would have more of an impact on oysters and their *Vibrio* load when emersed out of water, as they would not be shielded from the scattering and adsorption properties of the water column. It was envisioned that *V. aestuarianus* would be negatively impacted by UVR, although we could not predict the effect of UVR on OsHV-1 µVar.

2) **Can the diagnostic arsenal of *Vibrio* infections in bivalve shellfish be expanded upon to "blanket" screen and improve our understanding of *V. aestuarianus* infection in *C. gigas*?**

*Kett, G.F., Culloty, S.C., Jansen, M.A. and Lynch, S.A. (2022).*

*"Development of a sensitive polymerase chain reaction (PCR) and digoxigenin (DIG)-labeled in situ hybridization (ISH) for the detection of Vibrio bacteria in the Pacific oyster Crassostrea gigas." Aquaculture Reports, 22, p.100961.*

Metrics: Citations: 2. Readers: 157. Social media impacts: 19 (06/10/2022).

Two novel diagnostic tools were added to the arsenal: i) a generic *Vibrio* standard PCR, which is affordable, accessible, and useful as blanket screening tool, and ii) *in situ* hybridisation (ISH) that use the same primer pair to visualise and locate bacterial infections in host tissues via microscopy. This new method will aid an understanding of bacterial behaviour (migration within the host and tissue preference) under different environmental conditions. This tool would also serve as a confirmatory method when PCR detectability may be affected by experimental procedure e.g. UV exposure and cyclobutane pyrimidine dimer (CPD) formation.

Current methods used to detect and measure *Vibrio* pathogen occurrence and abundance are useful, these include already-existing PCR and qPCR protocols. However, no method for ISH existed that did not include the use of expensive and often hazardous fluorescent reagents and fluorescent microscopes which are specialised and costly. This research showed that by designing new tools for use in the detection and localisation of *Vibrio* infections, we can improve our understanding of *Vibrio* within *C. gigas* tissues. These new tools would improve our confidence in determining infection/detection discrepancies.

From this research we were able to determine that following UV exposure, *Vibrio* bacteria detection decreased, not from bacterial migration, nor from detection error due to CPDs, but from reduced number of viable bacterial cells within the oyster.

**3) What impact does supplemental UV-B radiation have on the health of *C. gigas* and the performance of *V. aestuarianus*?**

*Kett, G.F., Jansen, M.A., Culloty, S.C. and Lynch, S.A. (2022). "The impact of UV-B radiation on pacific oyster Crassostrea gigas health and pathogen Vibrio aestuarianus development." Journal of Experimental Marine Biology and Ecology, 555, p.151783.*

Metrics: Readers: 30. Social media impacts: 10 (06/10/2022).

Supplemental UV radiation had a demonstrably negative impact on the survival of *C. gigas* and on the performance of *V. aestuarianus*. This was most notable in smaller oysters, likely due to thinner shells, especially when emersed out of water. *V. aestuarianus* detection was lowered after UV exposure, although this effect was usually transient and detection levels returned to 'normal' within 2 – 3 days after the UV stressor was removed. Intensity of infection was severely reduced following UV exposure, suggesting mortality was due to UV rather than disease. Histological analysis showed the cellular impact of *V. aestuarianus* infection which included digestive gland atrophy, diapedesis, necrosis and deciliation.

This research provided further insight into *C. gigas* summer mortality episodes. Despite many previous investigations being carried out into this phenomenon, UV was not included as a potential factor in the mortality episodes or the related disease outbreaks. With this information we can approach future studies with a better understanding of potential factors driving *C. gigas* mortality at inter-tidal culture sites. We also know that *V. aestuarianus* is susceptible to UV-B radiation even when located within oyster tissues, this information

allows us to investigate the role of solar UV radiation as a natural disinfectant in intertidal or shallow water culture sites. This research has also shown that histological methods are indeed useful for visualising and characterising *Vibrio* infections in bivalve molluscs. Alongside the increasing use of molecular tools to quantify pathogen detection levels, histological and ISH tools can provide further information permitting a better understanding of this intricate host-pathogen relationship.

**4) Does grow-out shore height and emersion at a culture site influence *C. gigas* performance, pathogen development and partitioning?**

*Kett, G.F., Jansen, M.A., Culloty, S.C. and Lynch, S.A. (in prep.). "Investigating the effects of solar UV radiation on host performance and pathogen development using the Pacific oyster Crassostrea gigas and the intertidal environment as a model."*

Emersion (+4 hrs) negatively affected the survival of *C. gigas* in periods of extreme summer weather such as was experienced during this field trial experiment. High solar radiation (>2.4 kJ/m$^2$) and air temperature (>21 ºC) resulted in higher oyster mortality compared to those held lower on the shore and submerged for longer. In the Autumn months with less UV and temperature, mortality was higher in low shore oysters submerged underwater for longer periods. Pathogen partitioning was evident in this study, in high shore *C. gigas*, higher levels of OsHV-1 uVar and lower levels of *V. aestuarianus* were detected compared to *C. gigas* held on the low shore. Survivor selection screening bias was considered as a factor in this observation but given the low mortality overall on both shore heights following the initial

acclimation period (< 6%), this was not assumed to be the case, rather pathogens are likely reacting to the environmental and tidal conditions of the sites.

These findings contribute to the repertoire of *in situ* shore placement/emersion studies that have been carried out globally aiming to understand emersion effects on *C. gigas* performance. The *C. gigas* mortality results shown here can be both supported by and in contrast with existing literature. This demonstrates the value of conducting case-by-case studies for localised sites, given the variety of additional external biotic and abiotic factors which are likely affecting overall outcomes plus the genetics (disease resistant/not) of the *C. gigas* stocks being investigated. The pathogen partitioning findings are interesting as they further confirm our theoretical and lab-based hypothesis on *Vibrio* being negatively impacted by UV exposure. These results also shedding light on the impact of UV on OsHV-1 µVar, indicating that, like other herpesviruses, it may be activated by solar radiation. These findings should prove valuable to the shellfish aquaculture sector as they point to husbandry techniques which could be applied to reduce the impact of disease outbreaks and mortality events.

## 6.2 Recommendations for future research

### 6.2.1 Questions arising from this project

- *Is the impact of UVR highly dependent on the wavelength in question? Can UVR have a positive health impact on bivalve molluscs, for example through increased vitamin D production or immune pathway activation?*

It is important to further study this area as predicted changes to the atmospheric climate including cloud cover, aerosol, and ozone, as well as changes to the aquatic environment will affect the incident level of UV received by benthic and intertidal organisms (Bais *et al*, 2018). The energetic potential of UV is far greater in short-wave bands such as UV-B which would be expected to incite more genotoxic damage than longer wave UV-A which may be responsible for stimulating a positive immune response (Hart *et al*., 2011). Although outside the scope of this research project, this information would be useful in predicting host-pathogen responses to changes in environmental conditions and may allude to a immune-stimulating effect of sunlight exposure in aquatic invertebrates.

- *What aspects of pathogen biology drive the variation in pathogen response to UVR, as seen between V. aestuarianus and OsHV-1 uVar? What impact does UVR have on different pathogen groups?*

It is known that short-wave UV-C is used to inactivate pathogens (Acra, et al., 1990) and that different pathogen groups have different tolerances to shortwave UV-C (Fernández-Boo *et al.*, 2021). This information is useful for commercial settings where disinfection is a goal outcome. However, little is known about the variation in responses of pathogens to longer-wave, environmentally relevant UVR doses. The findings discussed throughout this thesis (Chapter 1, Chapter 5) indicate that pathogen prevalence and pathogenicity are affected by changes in environmental and tidal conditions. Future research would benefit from investigating how free-living and waterborne pathogens are impacted by natural solar UVR and the growing

conditions of the species of interest. This could allow for improved prediction of disease outbreaks given known reactions of pathogens to natural sunlight and would allow for an increased valuation of natural solar disinfection as an important ecosystem service.

- *How does solar UVR affect OsHV-1 µVar and the host-pathogen dynamic during summer mass mortality events?*

Viruses show great variation in response to UVR, OsHV-1 µVar can be deactivated by high-intensity shortwave UV-C in experimental conditions (Schikorski *et al.*, 2011). However, findings such as these are not always relevant to field conditions where longer-wave UV-B and UV-A are more active. Some herpesvirus species such as human herpes simplex virus (HSV) are known to be activated by solar UV radiation (Norval, 2006). In Chapter 5 of this study, OsHV-1 µVar was found to be more prevalent at high shore sites exposed to an additional 4 hours of aerial conditions including solar UV radiation, compared to low shore groups. It would be advisable to study this interaction, between UVR, OsHV-1 µVar and bivalve hosts, further, to better understand and predict mass mortality events particularly in cases associated with extreme summer weather.

- *Is the pattern of pathogen partitioning observed in C. gigas spat also present in C. gigas adult?*

Oyster disease susceptibility and resistance typically vary by life stage and by pathogen. It is commonly observed in the field that OsHV-1 µVar causes mortality in oyster spat while *V. aestuarianus* has a greater impact on adult oysters (Azéma *et al.*, 2016, Bookelaar, 2018). Throughout this research

project, only spat and juvenile oysters were used as per the experimental design. We found that *V. aestuarianus* infection prevalence and intensity was significantly reduced in spat oysters following UV exposure in both lab- and field-based experiments, with more notable effects on smaller oyster spat, thought to be related to the thinner nature of smaller oysters, allowing UV transmission. With regards to OsHV-1 µVar, the virus was not detected in Chapter 3 lab experiment but was detected in Chapter 5 field experiment. Low detection rates throughout may be attributed to the oysters being bred specifically for OsHV-1 µVar disease resistance. In Chapter 5 we found OsHV-1 µVar to be more prevalent on oyster seed held at the high shore. It would be interesting to investigate whether similar pathogen partitioning pattens would be observed in different oyster life stages and in non-disease resistant groups, and whether diploid and triploid oysters exhibit the same patterns. With this information, producers would be better equipped to manage husbandry practices such as layout design in times of disease outbreak to optimise performance and minimise mortality losses.

### 6.2.2 Experimental design recommendations

In addition, future experimental design could work towards improving the environmental relevance of lab-based UVR experiments and increasing the certainty of factor-variable responses in field-based UVR experiments. For lab-based experiments, it is recommended that future studies try to make use of natural solar UV in indoor-outdoor settings or utilise UV supplementation bulbs that mimic the gradually increasing intensity of the solar cycle via slowbrightening bulbs. This would reduce the ‘square curve’ effect which is seen with ‘0 – 100’ UV-emitting bulbs and rather would elicit a response from the host-pathogen system more similar to that observed in natural ecosystems.

Furthermore, lab-based experiments could attempt to mimic tidal flow and ebb with controlled water flow mechanisms. Again, this would improve the environmental relevance of experiments as opposed to comparing emersed and immersed conditions separately.

For field-based experiments, it would be advisable to design an experimental set-up which allows for the separation of UVR and temperature effects. As the two factors are closely linked, it proved difficult to discern the impacts statistically and so taking this into experimental design could be an effective way of measuring UV impacts individually. One way of achieving this would be to follow the direction of plant scientists conducting UVR research – experiments in this field are often conducted using plastic broadband or narrowband filters which allow the transmission of certain UV wavelengths while blocking others. Such materials include polyethylene, polycarbonate and cellulose acetate (Aphalo *et al.*, 2012, Boelen *et al.*, 2017). This type of experiment would allow researchers to investigate the impact of specific UV wavelengths on animal and pathogen biology while limiting the interfering effect of air temperature. However, this type of experiment design has proved difficult in aquatic and especially in dynamic tidal conditions where water pressure, wave action and sea salt residue affect the integrity and optical properties of the filters (Aphalo *et al*., 2012, Kett, G., pers. obs.).

Finally, future works would benefit from including longer datasets with more fine-scale physiological and environmental data assessment. UVR may induce sub-lethal effects on oyster immune functioning or affect oyster health indirectly through changes to plankton assemblages. *In situ* studies could also investigate interactions between UVR and additional environmental factors

such as ocean acidification, oxygen concentration, salinity, as well as external factors such as hazardous chemicals, microplastics and harmful algae blooms. It is recommended that future studies utilise climate model predictions and assess the potential for impacts of reduced UV levels given the recovery of ozone and predicted increases to cloud cover and decrease in water clarity associated with heavy rainfall patterns and the consequent nutrient-laden runoff into coastal habitats (Häder *et al*., 2015, Williamson *et al*., 2017, Bais, *et al*., 2018).

## 6.3 Commercial application of findings

- In future UV experiments, standardising, and sharing of best practices would be helpful to collate, cross-reference, and corroborate UVrelated animal health impact studies. In chapter 2 of this thesis, several complications were encountered when searching for and analysing UVR-animal impact studies. Studies varied in reporting dosages especially in UV wavelength and intensity, many studies did not specify the UV band applied lending to removal from the review as results could not be compared with other studies. Future studies which examine the impact of UV radiation would benefit from specifying whether the UVR source was natural or artificial, producing doseresponse models, providing the spectrum of intensities of each wavelength emitted by the source, and the optical characteristics of the experiment e.g., if underwater, the absorption and scattering properties of the water, and if in aerial conditions, the reflective properties of the holding chamber of the experiment.
- For pathogen surveillance monitoring and microbiome studies, the newly developed PCR can save time and money when looking at overall *Vibrio* levels in a population. This generic PCR for detecting

*Vibrio* can be applied in "blanket screening" detection assays which can allow for early detection of significant increases in *Vibrio* abundance which could be indicative of pathogen outbreaks or notable changes to microbiome structure.

- *In situ* hybridisation (ISH) is a useful tool which can be included in monitoring programmes to help identify infection pathways and pathological processes of *Vibrio* infections. ISH offers the ability to improve understanding of host-pathogen dynamics for example, how *Vibrio* behave when additional biotic or abiotic stressors are present.
- Husbandry practitioners can apply the results of Chapter 5 by realising and adjusting for the role of UV radiation in oyster spat mortality. It is advisable that when spat are first being laying on the shore during summer months, that a gradual acclimation period is allowed for so that oysters may become accustomed to the tidal fluctuations and relevant stressor exposures. Furthermore, starting smaller spat at lower growing heights and increasing immersion time may reduce stock mortality during extreme summer conditions of high temperatures and UVR.
- Oyster culture sites may also apply the findings in Chapter 5 to husbandry practices in terms of oyster spat placement during pathogen outbreaks. It was found that higher shore heights with increased emersion can avail of solar antibacterial disinfection when *Vibrio* outbreaks occur. However, caution should be taken here as OsHV-1 Var does not appear to be hindered by UVR exposure. Knowing which pathogens are present and proliferating is an important part of pathogen monitoring projects and as such, producers should be in communication with their local shellfish and seafood health monitoring

agency (for example, Marine Institute Fish Health Unit). Further research is recommended in this area to fully understand and predict the effect solar UVR has on OsHV-1 µVar in *C. gigas*.

## 6.4 Perspective

Shellfish production is seen as one of the most important tools in feeding a growing human population with minimal ecological impact (FAO, 2018). As the least carbon intensive source of protein, it is vital to ensure the industry remains viable in the face of climate impacts and emerging diseases. Oysters, along with other bivalve molluscs and algae species, are a crucial element of nature-based solutions to protect ecosystem functioning and coastal resilience whilst providing for a sustainable bioeconomy (Hynes *et al.*, 2022). The work carried out as part of this thesis feeds into this security by furthering our understanding of the drivers of *C. gigas* mass mortality episodes in order to better predict and adapt to their occurrence. An advanced understanding the host-pathogen-environment is key to a safe and predictable coastal ecosystem and an economically sustainable aquaculture industry. By ensuring a healthy, productive, and secure low-trophic aquaculture sector, we are directly feeding into numerous United Nations Sustainable Development Goals (UN SDGS), particularly Goal 14 Life Below Water by enriching coastal ecosystems and providing clean water and reef structures for biodiversity. Indirectly, this work supports SDG Goal 2 Zero Hunger, Goal 6 Clean Water and Goal 13 Climate Action by encouraging the sustainability of a low-impact, low-cost, highly accessible and highly nutritious food source that sequesters carbon and denitrifies water as it grows. Closer to home, *C. gigas* aquaculture provides direct employment for over 1,100 people in Ireland alone, bringing in over 40 million euros revenue annually (BIM, 2020). Disease outbreaks can decimate

stocks resulting in up to 80% mortality (EFSA, 2015). With each advance in knowledge, each new tool developed and each time those knowledge and tools are applied, studied, and built upon, further steps are made in the progression to a safe, secure, sustainable blue economy.

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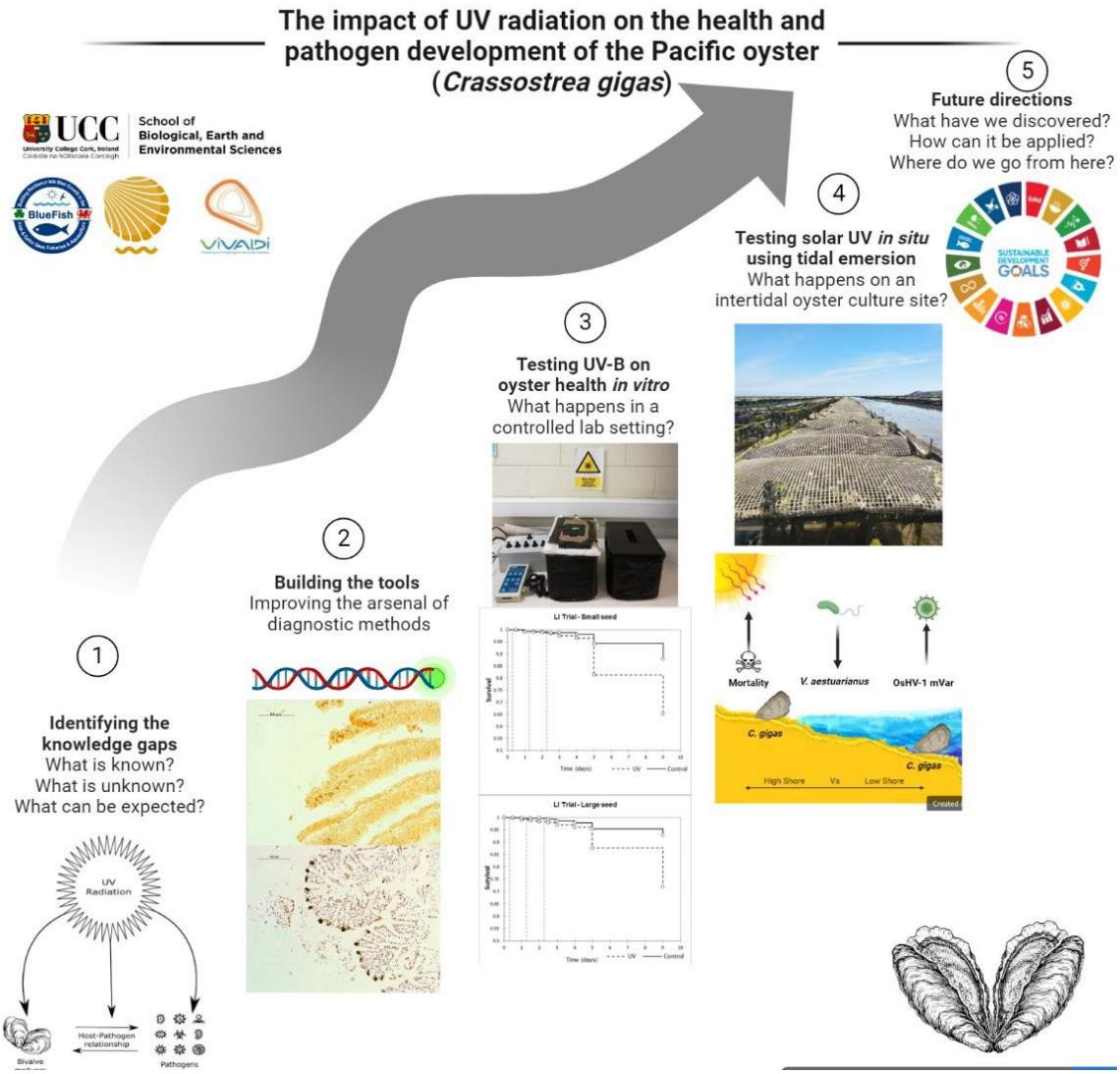



Figure 1. Summation of the research steps and their main findings throughout this project.

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## Supplementary Materials

### Chapter 4: The impact of UV-B radiation on Pacific oyster *Crassostrea gigas* health and pathogen *Vibrio aestuarianus* development.

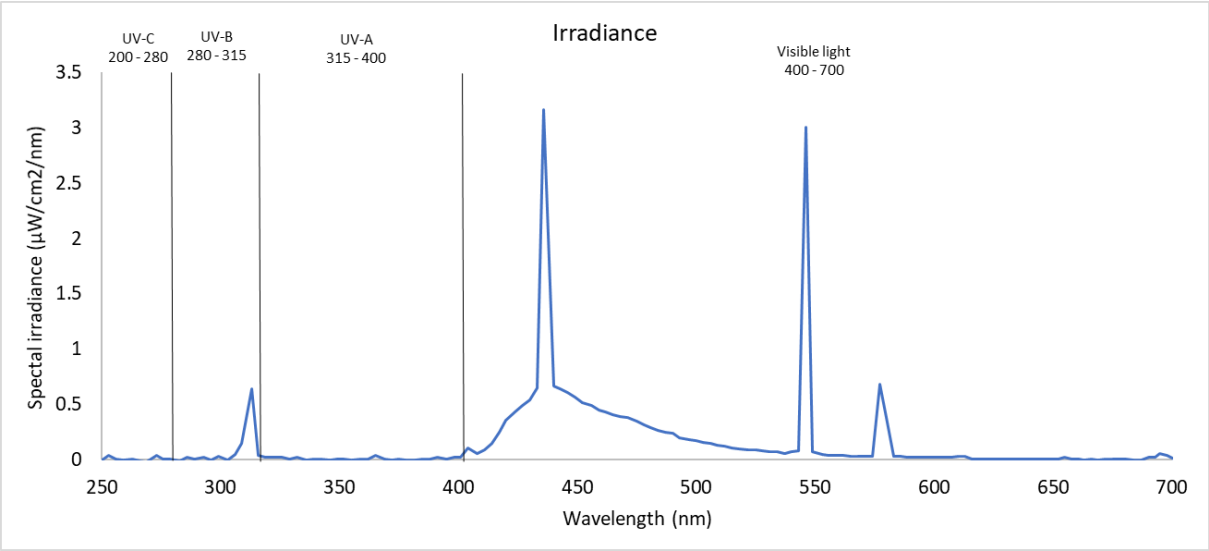



Figure 1. Wavelength spectrum of radiation emitted by used UV bulbs.

**Table S1. Mean (range) of oyster size groups as sampled in Trials 1 and 2**

| | Trial 1 - High intensity, underwater trial | | Trial 2 - Low intensity, air exposure trial | |
|---|---|---|---|---|
| **Oyster size group** | **Mean Length (range) (mm)** | **Mean Weight (range) (g)** | **Mean Length (range) (mm)** | **Mean Weight (range) (g)** |
| Small seed | 8.3 (3.6 – 12.4) | 0.17 (0.045 – 0.94) | 17.9 (9.1 – 26.8) | 0.81 (0.24 – 2.43) |
| Medium seed | 12.84 (8 – 20.1) | 0.43 (0.13 – 4.09) | 31.4 (20.18 – 41.6) | 2.81 (0.6 – 5.7) |
| Large seed | 46.3 (33.6 – 64.5) | 9.82 (6.06 – 16.5) | - | - |

1 **Table S2. Digestive gland atrophy scale**

| Score | Description |
|---|---|
| **0** | Normal wall thickness in most tubules, few tubules even slightly atrophied. |

| | |
|---|---|
| **1** | Wall thickness less than half of normal level, most tubules with some atrophy, some tubules still normal. |
| **2** | Wall thickness roughly half as thick as normal. |
| **3** | Wall thickness more than half normal levels, most walls heavily atrophied, some extremely thin (fully atrophied). |
| **4** | Walls extremely thin (100% atrophied), nearly all tubules affected. |

2

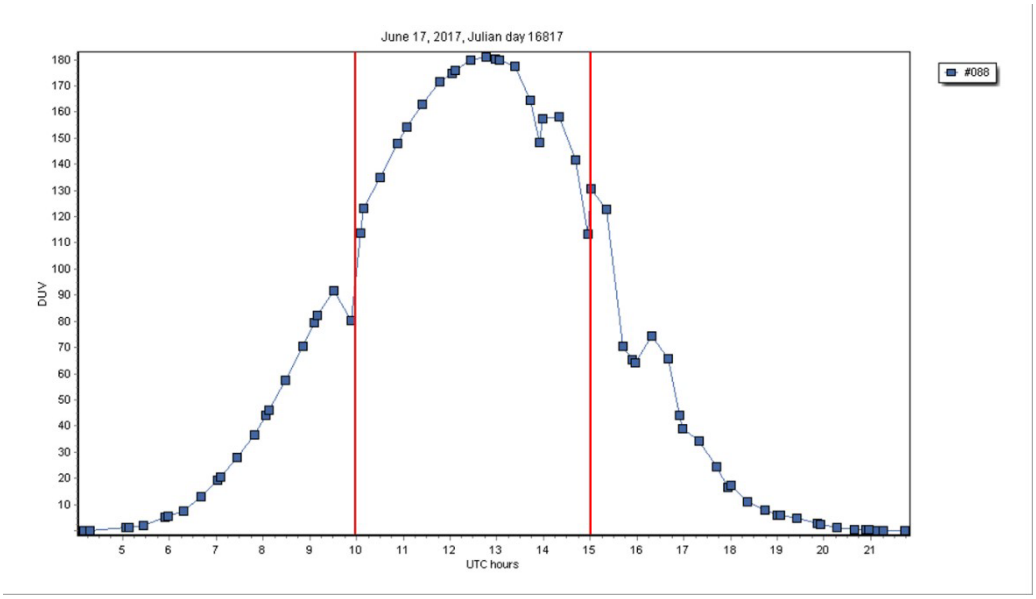
3

1 Chapter 5. Investigating the effects of solar UV radiation on host

2 performance and pathogen development using the Pacific oyster *Crassostrea*

3 *gigas* and the intertidal environment as a model.

4




Supplementary figure 1. Total daily incident UV typical of a clear sunny day in July at Valentia island observatory, Ireland. Red vertical lines delineate the period around solar noon used in this study which contains the highest intensity (>95% of DUV >70 $mW.m^2$) of total daily UV.

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## Modules undertaken during this thesis

PG6001 - **STEPS - Scientific Training for Enhanced Postgraduate Studies**. 5 Credits.

PG6017 - **Teaching and Demonstrating Skills for Biological, Earth and Environmental Sciences (BEES) Postgraduate Students**. 5 Credits.

PG6029 - **Skills in Public Engagement of Science**. 5 Credits.

BL6024 - **Quantitative Skills for Biologists using R**. 10 Credits.

**Innovating Field Trips.** 10 ECTS. Erasmus+, CERES project, University College Cork

**Climate Leadership Journey**. 10 ECTS, level 9. European Institute of Innovation and Training, Climate-KIC.

## Additional publications

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**Kett, GF**, Notaro, DA, Higgs, BM, Theias, A, Hadre, E, Bamberger, A, Blum, A. (2019). "**Learning by doing: an international, interdisciplinary experiment using peer-based learning in an outdoor laboratory**" Learning Connections: Spaces, People, Practice (2019), 92 – 97.

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## Conference Proceedings

### Oral presentations

- Kett, G. F., Lynch, S.A. Culloty, S. C. "The impact of UV-B radiation on the health of Pacific oysters (*Crassostrea gigas*)". Environmental

Association of Ireland – Environmental Researchers Colloquium (ENVIRON, 2018). Cork Institute of Technology, Cork, Ireland. 26th – 28th March 2018. **Awarded Best Aquatic Presentation,*

- Kett, G. F., Lynch, S.A. Culloty, S. C. "Climate Change & Shellfish Aquaculture" UCC Climate Lab Open Day at University College Cork, Cork, Ireland 24th April 2018.
- Kett, G. F., Lynch, S.A. Culloty, S. C. "Effects of changing UV-B radiation on the health of the Pacific oyster Crassostrea gigas". BES Aquatic Ecology – "Fishes of the Future. Imperial College London, UK. 3rd September 2018.
- Kett, G. F., Jansen, M.A.K., Lynch, S.A. Culloty, S. C. "How does UVB affect Pacific oysters and the development of their pathogens?" *UV4Plants workshop "UV-B and Climate Change", Cork, Ireland.* 15th – 16th April 2019
- Kett, G. F., Lynch, S.A., Jansen, M.A.K., Culloty, S. C. "The impact of UV radiation on Pacific oyster health and development of *Vibrio aestuarianus* and Ostreid herpesvirus-1". EAFP 19th International Conference on Diseases of Fish and Shellfish. Porto, Portugal. 9th – 12th September 2019.
- Kett, G. F., Lynch, S.A., Jansen, M.A.K., Culloty, S. C. "The impact of UV radiation on Pacific oyster health and pathogen development" Aquaculture Europe. Online. 14th April 2020.

## Poster presentations

- Kett, G. F., Lynch, S.A., Jansen, M.A.K., Culloty, S. C. “Impact of biotic and abiotic stress on the Pacific oyster (*Crassostraea gigas*) in Ireland” - Pacific oyster workshop “Invasions of the Pacific oyster”. NIOZ - Royal Netherlands Institute for Sea Research, Texel, The Netherlands). 1st – 2nd November 2017.
- Kett, G. F., Lynch, S.A., Jansen, M.A.K., Culloty, S. C. “Impact of UV-B radiation on the Pacific oyster (*Crassostraea gigas*) aquaculture in Ireland”. National Shellfisheries Association 110th Annual Meeting. Seattle, USA. 18th – 22nd March 2018 “”
- Kett, G. F., Jansen, M.A.K., Lynch, S.A., Culloty, S. C. *"Connecting Academia with Industry for improved Aquatic Animal Health”.* EAFP UK & Ireland Branches Third Meeting, Galway, Ireland. 11th - 12th September 2018.
- Kett, G. F., Lynch, S.A., Culloty, S. C., Jansen, M.A.K. “Solar radiation: Abiotic stressor or vital disinfectant?” Irish Ecological Society 2nd Ecology & Evolution Ireland Conference, Galway, Ireland.10th – 12th January 2019.

**FIN**