Oyster disease in a changing environment: decrypting the link between pathogen, microbiome and environment

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Abstract

Shifting environmental conditions are known to be important triggers of oyster diseases. The mechanism(s) behind these synergistic effects (interplay between host, environment and pathogen/s) are often not clear, although there is evidence that shifts in environmental conditions can affect oyster immunity and, pathogen growth and virulence. However, the impact of shifting environmental parameters on the oyster microbiome and how this affects oyster health and susceptibility to infectious pathogens remains understudied. In this review, we summarise the major diseases afflicting oysters with a focus on the role of environmental factors that can catalyse or amplify disease outbreaks. We also consider the potential role of the oyster microbiome in buffering or augmenting oyster disease outbreaks and suggest that a deeper understanding of the oyster microbiome, its links to the environment and its effect on oyster health and disease susceptibility, is required to develop new frameworks for the prevention and management of oyster diseases.

Key words: aquaculture, climate change, pathogen, oyster disease, microbiome

1.0 Introduction

Oysters are filter-feeding bivalve molluscs that inhabit estuarine and coastal environments. They encompass a number of different species, many of which are heavily farmed for human consumption, supporting valuable aquaculture industries. In 2005, global bivalve aquaculture was responsible for 13.6 million metric tons of production, valued at $1.82 billion USD, with oysters responsible for 4.8 million metric tons of production (Pawiro, 2010). Four oyster species, namely, *Crassostrea gigas* (the Pacific oyster), *Saccostrea glomerata* (formerly *S. commercialis* and also known as the Sydney rock oyster), *Ostrea edulis* (the European flat oyster) and *Crassostrea virginica* (the Eastern oyster or American cupped oyster) are amongst the most heavily cultivated historically and/or currently across different regions of the world.

Infectious diseases have become a major obstacle for the successful growth and sustainability of oyster aquaculture industries, with a range of diseases having severe detrimental effects on oyster yields. For example, historical outbreaks of *C. virginica* diseases contributed to hundreds of millions of dollars in economic losses (Ewart and Ford, 1993). While diseases of *S. glomerata* in Australia, and *O. edulis* in Europe, have also severely diminished their production capacity (René Robert and O'Mahoney, 2013; Schrobback et al., 2015; FAO, 2016c). Another species of oyster, *Crassostrea angulata*, was extensively cultivated in France

prior to the 1970's before the industry was completely wiped out as a consequence of infectious disease outbreaks, resulting in this species being replaced by imported *C. gigas* (Roch, 1999). These few examples highlight just some of the impacts that infectious diseases have had on global oyster cultivation.

Since oysters are typically reared in uncontrolled and often dynamic coastal and estuarine environments, it is often difficult to predict, manage and control infectious disease outbreaks. Management strategies designed to control the spread of pathogens are further constrained by the ability of marine pathogens to rapidly spread over large distances, due to reduced dispersion barriers in aquatic habitats, relative to terrestrial environments (McCallum et al., 2003). Increasing evidence is showing that oyster diseases have strong environmental drivers such as temperature. Notably, outbreaks are often more severe closer to the tropics (Leung and Bates, 2013) likely due to the preference of many pathogens to grow in warmer waters (Leung and Bates, 2013), or the exertion of temperature stress as oysters reach their thermal limits (Bougrier et al., 1995). Within the context of temperature driven disease outbreaks, the implications of climate change (i.e. warming waters in non-tropical areas) on pathogen spread, transmission and virulence are a concern for future food security (Harvell et al., 2002). Specific examples supporting this concern include warming oceans driving the geographic spread of *Perkinus marinus*, the parasite responsible for dermo disease in *C. virginica* (Ford, 1996; Cook et al., 1998) and, the enhanced replication and transmission of the *C. gigas* disease-causing herpesvirus OsHV-1 and growth of *Vibrio* species in *C. gigas* tissues at warmer temperatures (Petton et al., 2013; Renault et al., 2014).

The disease process has traditionally been viewed as a 'one pathogen one disease' system, a classical view pioneered by Robert Koch now known as Koch's postulates (Koch, 1884; Löffler, 1884). Since that time, our understanding of infectious disease processes has evolved from a 'classical view' to one of an 'ecological view', in which multiple factors contribute to or amplify the disease process (Wilson, 1995). As with most infectious processes, many oyster diseases appear to be complex and often proceed as a result of a shift or fracture in the interplay between environmental (e.g. temperature, salinity, pH, nutrients) and biological factors, including oyster fitness, the oyster microbiome, the abundance and virulence of external pathogens and their potential vectors (e.g. phytoplankton). Detangling the causative mechanisms of disease from this complex "interactome" (the suite of biotic and abiotic factors that participate in disease processes) is not trivial – in particular, little information is known regarding the role of the microbiome in disease protection or susceptibility. In order to develop

more effective strategies for managing infectious outbreaks within oyster harvesting practices, a new understanding of the interactome and the role of the microbiome is necessary. In this review, the major diseases affecting oyster aquaculture will be covered and in particular, the potential synergistic importance of the oyster microbiome and local environmental parameters in these infectious outbreaks will be evaluated.

2.0 The oyster life cycle, anatomy and distribution

In this section, we will focus on four major commercial oyster species, including *C. gigas*, *S. glomerata*, *O. edulis* and *C. virginica*, which are harvested in a number of regions across the globe **(**Figure 1). *C. gigas* is the most widely grown species, with commercial industries in the USA, Canada, Mexico, Chile, Argentina, South Africa, Namibia, China, Japan, Australia and a number of European countries, in particular France (FAO, 2016a). *C. virginica* is grown exclusively in the USA, Canada and Mexico (FAO, 2016b), while *S. glomerata* is only grown in Australia (FAO, 2016d). The limited production of *O. edulis* is restricted to several European nations, the USA, and South Africa (FAO, 2016c).

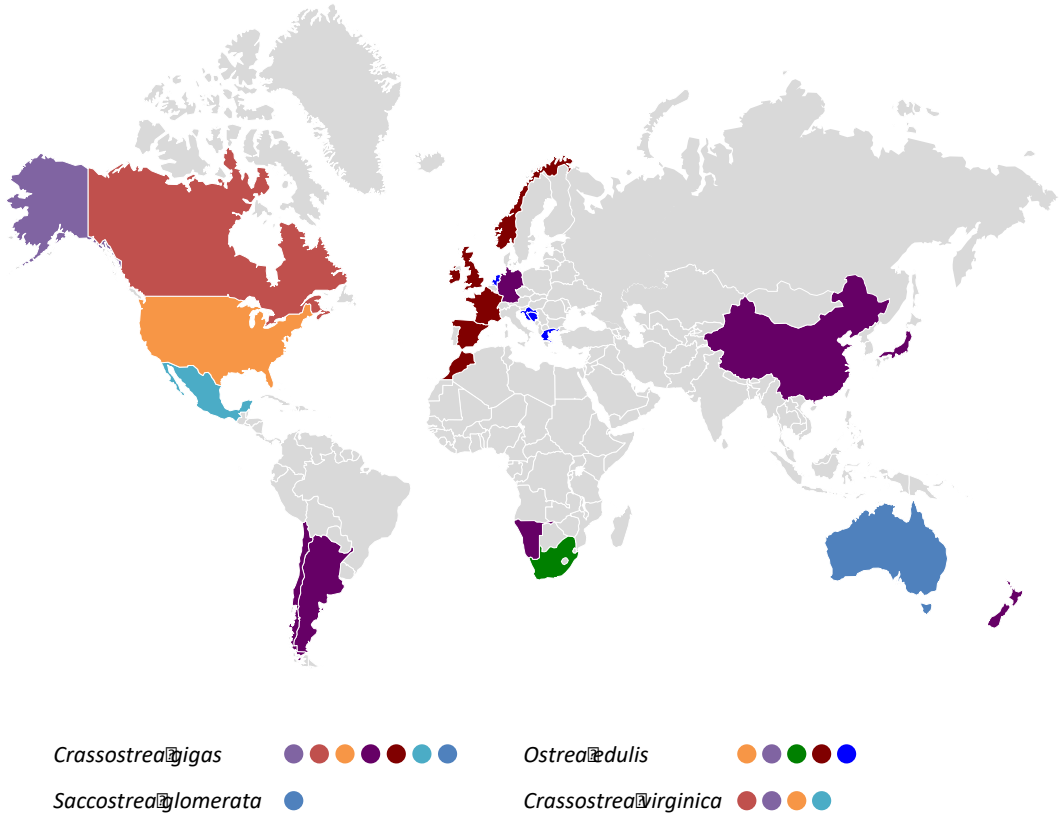

Figure 1: Global cultivation of four oyster species. *C. gigas* is grown in the largest number of countries, spanning North and South America, Western Europe and Australia. While *S. glomerata* is only grown in Australia. *C. virginica* is exclusively grown in North America, whereas *O. edulis* is grown in the USA, a number of European countries and South Africa.

There are numerous microbial and viral diseases that can infect one or more stages of the oyster life cycle. Across all species of oysters, the general oyster life cycle is relatively consistent (Figure 2). The life cycle begins with spawning, which is dependent on temperature and location (Fujiya, 1970; Wallace, 2001; FAO, 2016a; d; c). Following spawning events, fertilisation occurs, resulting in the development of a free-swimming planktonic larva (trochophore) (Wallace, 2001). At this stage, the oyster larvae are particularly vulnerable to infection by mostly viral and bacterial pathogens (Hine et al., 1992; Luna-González et al., 2002; Elston et al., 2008). After settlement on a hard surface, metamorphosis occurs developing into a juvenile oyster form called spat (Wallace, 2001). Similar to the larval form, spat are prone to infection by bacterial and viral pathogens (Waechter et al., 2002; Friedman et al., 2005). After 12-40 months of growth, the spat grows into a commercially harvestable adult oyster. Relative to the earlier forms, adult oysters are more resistant to viral infection (Dégremont, 2013) with infections from protozoan parasites more likely (Friedman and Perkins, 1994; Green and Barnes, 2010).

The oyster possesses a number of specialised tissues and organs to help it survive in its environment (Figure 2). The gills draw in water and directs the collected food particles (such as phytoplankton) to the palps, which sort the food particles before they enter the digestive system. The digestive gland is a common site for protozoan parasite infection often culminating in oyster starvation (Alderman, 1979; Ewart and Ford, 1993; Kleeman et al., 2002). The mantle acts as a sensory organ to initiate opening and closing of the shell, and forms the oyster's shell (Quayle, 1988; FAO, 2016e). Shell infections are observed from some bacterial species, resulting in mantle lesions and abnormal shell deposits (Bricelj et al., 1992). The heart is responsible for circulating the oyster hemolymph, a clear fluid that acts as the oyster 'blood' and contains cells called hemocytes with immune functions (Bachere, 1991). Previous research has indicated that viral pathogens are able to invade and replicate within these hemocytes (Morga et al., 2017). Finally, the gonad represents the reproductive system, which involves the production and release of gametes (spawning) (FAO, 2016e).

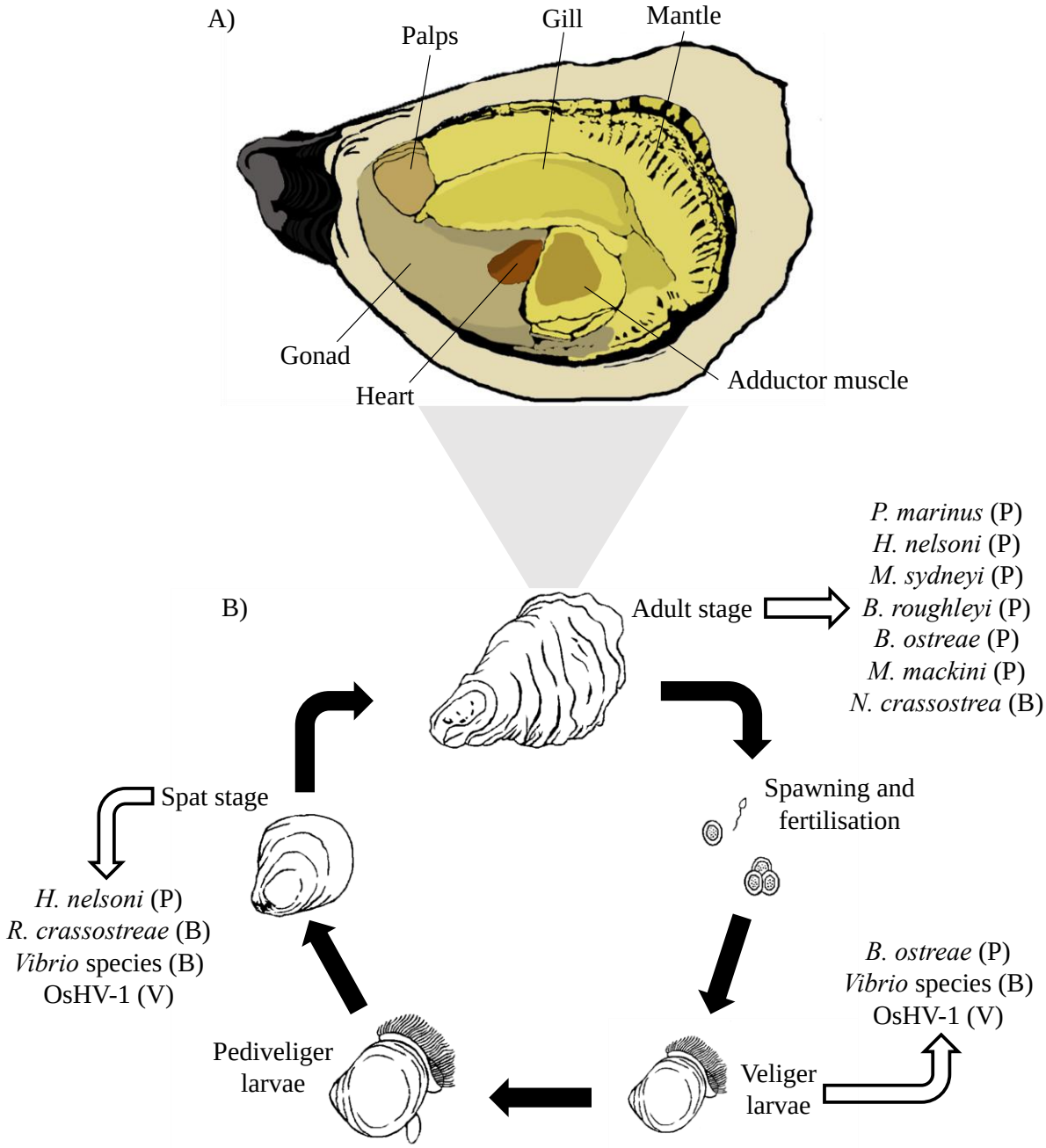

Figure 2: The basic anatomy A) and generalised life cycle of oysters B). Oyster pathogens infect various stages of the oyster life cycle. Bacterial and viral pathogens typically infect the spat and larval stages, while the protozoan parasites dominantly infect the adult stages. Black arrows depict the life cycle progression. Black hollow arrows highlight the known pathogens of commercial oysters at each life stage. (P), (B), and (V) represent parasites, bacteria, and viral agents respectively. Image produced by Sarah J Iwanoczko

3.0 Oyster immunology

Oysters are filter feeders, filtering around 163 litres per day (Riisgård, 1988) and given that the average litre of seawater contains more than a billion microbes, oysters are constantly exposed to a large number of microorganisms present in seawater. In order to combat pathogenic microorganisms, the innate immune system of the oyster is its primary defence (Schmitt et al., 2012a). This immunity is primarily facilitated by hemocytes (Figure 3), and molecules/proteins contained in both the hemolymph and epithelial mucus secretions (Cheng and Rodrick, 1975; Itoh and Takahashi, 2008; Pales Espinosa et al., 2014; Allam and Pales Espinosa, 2016).

The oyster hemolymph is not sterile, with low concentrations (10^2-10^5 cells mL^{-1}) of bacteria, primarily from the genera *Vibrio, Pseudomonas, Aeromonas* and *Alteromonas,* which appear to naturally reside within the oyster circulatory system (Olafsen et al., 1993; Garnier et al., 2007). This raises the questions of how hemocytes differentiate between pathogens and "natural" inhabitants and may be related to the function of pattern recognition receptor proteins (e.g. peptidoglycan recognition proteins) and antimicrobial peptides (AMPs) produced by these cells. Pattern recognition receptors are produced by oyster epithelial cells and hemocytes (Itoh and Takahashi, 2008) and when stimulated (by microbial products such as peptidoglycan), activate hemocytes, allowing them to migrate to the invasion site and express AMPs for a rapid and effective defence against invading microbes (Schmitt et al., 2012b). Additionally, the epithelial layer constitutively expresses a number of AMPs to further reduce microbial loads (Schmitt et al., 2012b).

Pathogens bypassing these initial defence strategies face phagocytosis by the circulating hemocytes in the hemolymph. Phagocytised pathogens (Canesi et al., 2002) are subsequently exposed to reactive oxygen species (ROS), enzymes and AMPs within the hemocyte (Labreuche et al., 2006a; Schmitt et al., 2012b). However, some bacterial and protozoan parasites are able to subvert intracellular degradation, effectively evading the oyster immune response (Schmitt et al., 2012c). This is primarily facilitated by the suppression of (ROS) generation, or reduced phagocytosis by the hemocytes (Schott et al., 2003; Labreuche et al., 2006b).

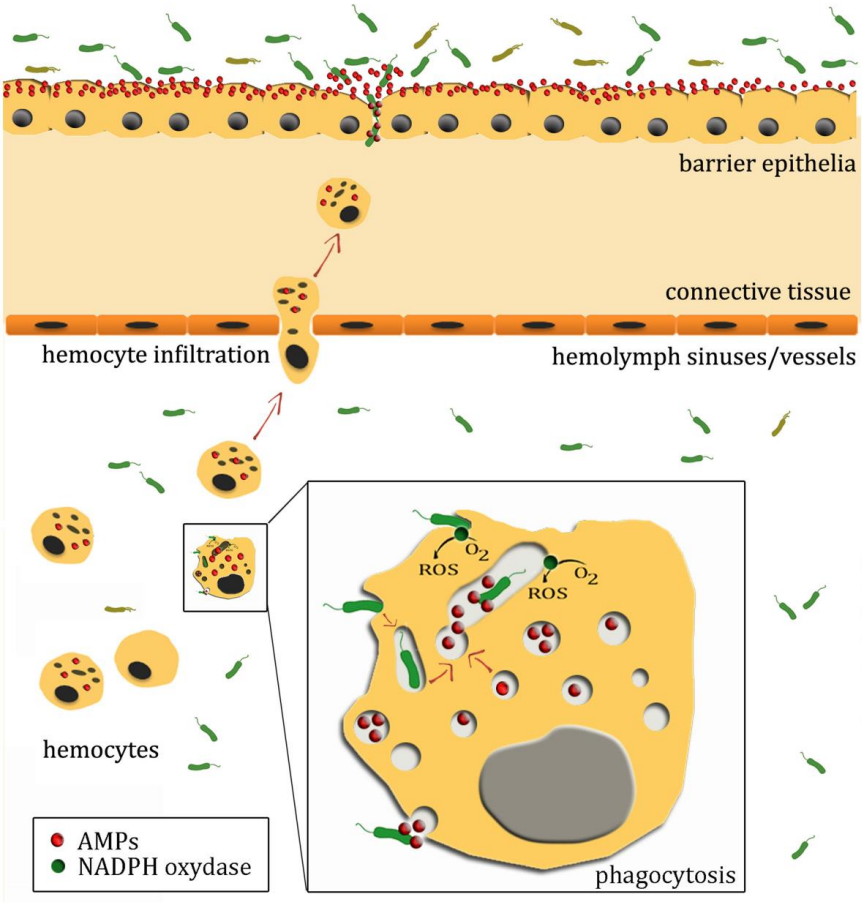

Figure 3: An overview of the oyster cellular immune response (Schmitt et al., 2012c), published by Frontiers in Microbiology. Invading pathogens must first bypass the epithelial layer, which produces antimicrobial peptides (AMP; red circle). Following this, the circulating hemocytes in the hemolymph engulf the microbial pathogens. They are then exposed to reactive oxygen species (ROS), which are produced by either NADPH oxidase (green circle) or the mitochondria, and antimicrobial proteins such as lysozyme and AMPs.

4.0 Diseases affecting oysters of economic importance

There are a number of well-characterised microbial diseases affecting several different oyster species. A summary of the known oyster diseases for each species is provided in Table 1.

186 Table 1 Diseases of economically important oyster species, their affected life stage and the pathology seen for each disease.

Oyster species	Disease/pathogen (agent)	Affected oyster stage	Pathology	Geographical distribution	Mortality range (%)	References
The Eastern oyster (*Crassostrea virginica*)	Dermo/*Perkinsus marinus* (Protozoan)	Adult	Tissue lysis, blockage of circulatory system	USA East Coast	20-85	(Andrews and Hewatt, 1957; Ford, 1996)
	MSX/*Haplosporidium nelsoni* (Protozoan)	Spat and adult	Epithelium infection, respiratory and digestive impacts	USA East Coast	33-95	(Haskin et al., 1966; Ford and Haskin, 1982; Ewart and Ford, 1993)
	ROD/*Roseovarius crassostreae* (Bacterium)	Spat	Mantle lesions, conchiolin deposits, tissue degradation	USA East Coast	54-75	(Bricelj et al., 1992; Boardman et al., 2008)

Sydney rock oyster (*Saccostrea glomerata*)	QX/*Marteilia sydneyi* (Protozoan)	Adult	Digestive tubule destruction, starvation	Australian East Coast	22-99	(Kleeman et al., 2002; Nell and Perkins, 2006)
	Winter Mortality/*Bonamia roughleyi* † (Protozoan)	Adult	Connective tissue disruption, ulcers, impaired muscle contractions, necrotic tissues	Australian East Coast	9-52	(Roughley, 1926; Mackin, 1959; Farley et al., 1988; Smith et al., 2000)
European flat oyster (*Ostrea edulis*)	Marteiliosis/*Marteilia refringens* (Protozoan)	‡	Digestive gland infection, impaired growth, starvation	France, Spain, Portugal and Greece	50-90	(Alderman, 1979; Virvilis and Angelidis, 2006; Bower, 2011; López-Sanmartín et al., 2015)

	Bonamiasis/*Bonamia ostreae* (Protozoan)	Adult, larvae	Gill and mantle lesions, parasite resides within hemocytes	France, Spain, England, Denmark, the Netherlands, USA West Coast	40-80	(Balouet et al., 1983; Elston, 1986)
Pacific Oyster (*Crassostrea gigas*)	Denman Island disease/ *Mikrocytos mackini* (Protozoan)	Adult	Green pustules, ulcers and abscesses on oyster tissues	USA Northwest Coast and Canadian Southwest Coast	17-53	(Quayle, 1961; Farley et al., 1988; Elston et al., 2015)
	Nocardiosis/*Nocardia crassostreae* (Bacterium)	Adult	Green pustules and lesions on oyster tissues	USA Northwest Coast and Canadian Southwest Coast	47-50	(Friedman et al., 1991)

	Vibriosis (Bacillary necrosis)/ *Vibrio* spp. (Bacterium)	Larvae, spat	Abnormal swimming, necrosis, lesions	Worldwide	76-100*	(Jeffries, 1982; Sugumar et al., 1998; Waechter et al., 2002; Elston et al., 2008)
	Pacific Oyster Mortality Syndrome/OsHV-1 and OsHV-1 µ variant (Virus)	Larvae, spat	Lesions and cells with viral inclusions and hypertrophied nuclei. Reduced feeding and impaired swimming in larvae	USA East Coast, Australia, New Zealand, France, Sweden and Norway	40-100	(Hine et al., 1992; Friedman et al., 2005; Segarra et al., 2010; Jenkins et al., 2013; Keeling et al., 2014; Mortensen et al., 2016)
	Summer Mortality/Unknown or multifactorial§	All stages	ill defined, characterised by high level mortalities during the warmer months	USA, France, Australia, Japan, Germany, Ireland, Sweden and Norway	30-100	(Mori, 1979; Soletchnik et al., 2005; Burge et al., 2007; Garnier et al., 2007; Malham et al., 2009)

†The aetiological agent of winter mortality may not be *Bonamia roughleyi*.

‡Age not reported, likely adult oysters are affected by marteiliosis as seen in QX disease.

§While no definite aetiological agent has been found, OsHV-1 and a number of *Vibrio* spp. have been associated with this disease usually during periods of host-stress (e.g. reproductive or heat stress).

*Depending on the *Vibrio* strain and bacterial concentration used.

4.1 Parasitic aetiological agents

Parasitic disease outbreaks have historically led to catastrophic losses of oysters, and large economic impacts. Dermo (also known as perkinsosis) and MSX are caused by the protozoan parasites *Perkinsus marinus*, and *Haplosporidium nelsoni* respectively (Mackin et al., 1950; Haskin et al., 1966). Specifically, historical outbreaks of dermo affecting *C. virginica* have contributed to hundreds of millions of dollars in economic losses (Ewart and Ford, 1993). Both dermo and MSX are responsible for extensive annual mortality outbreaks, particularly along the east coast of America (Encomio et al., 2005). For *S. glomerata*, Queensland unknown disease (QX) is caused by the protozoan parasite, *Marteilia sydneyi* (Anderson et al., 1994; Kleeman et al., 2002), while the aetiolgical agent of *S. glomerata* winter mortality is unclear with conflicting morphological, histological and molecular evidence from different laboratories (Carnegie et al., 2014; Spiers et al., 2014). These two diseases have reduced cultivation in some Australian estuaries by as much as 97% (Nell and Perkins, 2006; O'Connor et al., 2008; Dove et al., 2013b). QX disease has been particularly harsh with mortality rates as high as 85-95% (Anderson et al., 1994; Bezemer et al., 2006). The decline of the *O. edulis* industry in Europe has been attributed to two parasitic diseases, marteiliosis (also known as Aber disease) and bonamiasis (René Robert and O'Mahoney, 2013), caused by *Bonamia ostreae* and *Marteilia refringens* respectively (Alderman, 1979; Balouet et al., 1983; Elston, 1986).

4.1.1 Disease process of parasites

Parasitic diseases are chronic, typically taking weeks or months to kill their host through disruption of different tissue(s) usually causing effects such as oyster starvation, and/or tissue lysis (Andrews and Hewatt, 1957; Haskin et al., 1966; Balouet et al., 1983; Adlard and Ernst, 1995; Hervio et al., 1996). This section will review what is known about parasitic infections of oysters including the oyster tissue(s) where infection is initiated, the process(es) by which parasites move to other tissues/sites in the oyster and, process(es) that lead to oyster death.

Of the various oyster parasites, the point/site of infection can vary and include the gill and palps for *M. sydneyi* (Kleeman et al., 2002), and the mantle epithelium for *P. marinus* (Allam et al., 2013). However, for the remaining oyster parasites (*H. nelsoni*, *M. refringens*, *B. ostreae*, and *M. mackini*), the site(s) of infection are unknown and is an area that requires additional research. Despite this, gill infections are commonly observed for these parasites (Haskin et al., 1966; Balouet et al., 1983; Farley et al., 1988; Kleeman et al., 2002; Ragone Calvo et al., 2003;

Carnegie and Burreson, 2011), indicating that oyster filter feeding is an important process for the transmission of the parasite into the oyster with the gills possibly acting as the point of infection.

Following initial infection, subsequent dissemination to specific tissues or cells varies depending on the infecting parasite, with hemocytes, the digestive gland and connective tissue known targets. *P. marinus* and *B. ostreae* are phagocytosed by the circulating hemocytes (Balouet et al., 1983; Schott et al., 2003), and are both able to survive the process through degradation or preventing the formation of toxic reactive oxygen species inside the hemocyte (Schott et al., 2003; Morga et al., 2009). These parasites are able to proliferate within the hemocyte and use them as a vehicle to spread throughout the oyster (Montes et al., 1994; Perkins, 1996), resulting in the lysis of various host tissues and/or blockage of the oyster circulatory system thus culminating in mortality (Andrews and Hewatt, 1957; Balouet et al., 1983; Choi et al., 1989; Encomio et al., 2005). For the two *Marteilia* parasites, *M. sydneyi* and *M. refringens,* both lead to an infection of the digestive gland resulting in disrupted growth and impaired nutrient uptake leading to oyster starvation and mortality (Alderman, 1979; Camacho et al., 1997; Kleeman et al., 2002; Green et al., 2011). Destruction of the digestive gland and tubules is also observed for oysters infected with *H. nelsoni* (Ford and Haskin, 1982), but it is not clear whether the parasite also affects nutrient uptake similar to the *Marteilia* parasites. While it is known that systemic dissemination of *M. sydneyi* cells follows on from the initial gill and palp infection (Kleeman et al., 2002), it is unclear whether *M. refringens* and *H. nelsoni* also disseminate towards the digestive gland/tubules from an initial infection site, or whether the infection is initiated in the digestive gland/tubules. Connective tissue cells (cells between organ tissues) of the oyster are infected by *M. mackini* causing mortality through tissue disruption and necrosis (Hervio et al., 1996; Bower et al., 1997). This process produces characteristic green pustules, ulcers and abscesses on several different oyster tissues (Figure 4) (Farley et al., 1988; Hervio et al., 1996).

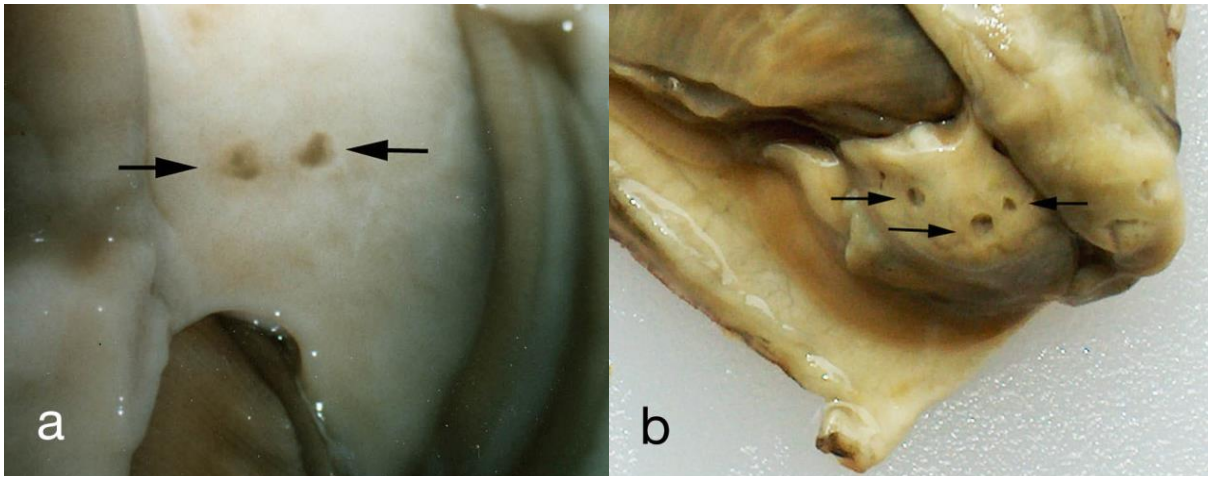

Figure 4: Ulcerated lesions (black arrows) on the labial palps of *Crassostrea gigas* characteristic of Denman Island Disease (Elston et al., 2015), published by Diseases of Aquatic Organisms, © Inter-Research 2015.

Since the aetiological agent(s) of winter mortality is still being debated (Spiers et al., 2014), the disease process remains poorly understood. Spiers et.al. (2014) carried out a longitudinal study with the aim of determining the aetiological agent of winter mortality. While the presence of a *Bonamia* spp. was confirmed by PCR, the occurrence of this parasitic organism was quite low (3% of all samples) and the 18S rRNA sequence of the observed protozoan was closely related to another organism, *B. exitiosa* which has previously been identified in *S. glomerata* (Carnegie et al., 2014) but not in association with clinical disease. The low prevalence of *Bonamia* spp. DNA in the Spiers et al. study was inconsistent with the high prevalence of pathological observations. Similarly, no *Bonamia* spp. was found within the lesions of the oysters (Spiers et al., 2014). While this research suggests that another organism may be causing or perhaps working with *Bonamia* spp. in winter mortality, this study only observed a 10% total mortality over the entire study period, which is not an extensive outbreak. As a result, further studies are required to elucidate the aetiological agent(s) of winter mortality before further research on the disease process can be elucidated.

4.1.2 Environmental reservoirs and transmission of infectious parasites

For the majority of infectious parasites, the environmental reservoir and details of transmission to and between oysters is not completely understood. On reservoirs, it is unknown whether the parasite is residing in the environment (i.e. the water column or in sediments), or whether an intermediate host is acting as an environmental reservoir. It may also be possible that the parasite is using the intermediate host for maturation and then residing in another unknown

organism. For example, *M. sydneyi* spores are only able to survive in the marine environment for up to 35 days, which is inconsistent with the yearly cycle of QX disease outbreaks (Wesche et al., 1999). It is therefore likely that an intermediate host exists as a reservoir of the parasite. Recent evidence suggests that *M. sydneyi* is present within the intestinal epithelium of the marine worm *Nephtys australiensis* and it has been proposed that this organism may act as a reservoir for *M. sydneyi* or may be critical for the maturation and transmission of *M. sydneyi* (Adlard and Nolan, 2015). Therefore, further research is necessary to determine where these parasites reside, and for those parasites with intermediate hosts, whether their intermediate host may act as that reservoir.

In regards to transmission, parasites can either be transmitted directly or via an intermediate. Direct transmission of parasites between infected and naïve oysters has been observed for dermo, bonamiasis, and Denman island disease (Elston, 1986; Quayle, 1988; Ewart and Ford, 1993; Hervio et al., 1996). While the causative agents of MSX, QX, and marteiliosis require an intermediate host(s) for the maturation and transmission of the parasite.

For those directly transmitted parasites, *P. marinus* is shed into the water column from infected oyster hosts, which can then be ingested by neighbouring oysters (Ewart and Ford, 1993). Similarly, only cohabitation with infected oysters is necessary for the transfer of *B. ostreae* and *M. mackini* to naïve hosts (Elston, 1986; Quayle, 1988; Hervio et al., 1996). The larvae of *O. edulis* can also be infected with *B. ostreae*, potentially allowing them to act as a reservoir of the parasite in the environment (Arzul et al., 2011).

For those parasites with no direct transmission, early laboratory-based studies were unsuccessful in transmitting *H. nelsoni* to uninfected oysters through co-incubation with infected oysters (Ewart and Ford, 1993). Later studies have demonstrated that an intermediate carrier capable of penetrating 1 mm^2 filters is required for transmission to naïve oysters (Sunila et al., 2000). Similarly, while field studies investigating the transmission of *M. refringens* into *O. edulis* demonstrated that the parasite was transmissible through cohabitation of uninfected with infected oysters or by deploying uninfected oysters in areas known to contain the pathogen (Berthe et al., 1998), laboratory-based cohabitation experiments and inoculations were insufficient to cause infections (Berthe et al., 1998). Later studies have identified two copepod species, *Paracartia grani* and *Paracartia latisetosa*, harbouring *M. refringens* and are implicated in the transmission of this parasite (Audemard et al., 2002; Arzul et al., 2014). This is similar for *M. sydneyi*, in which before an infected oyster dies, almost all of the *M. sydneyi*

sporonts (Figure 5) are shed into the environment (Roubal et al., 1989). However, direct transmission studies have been unable to transmit the parasite to naïve oysters (Lester, 1986). Likely the intermediate host, *Nephtys australiensis*, and possibly other unknown hosts, are needed to transmit *M. sydneyi* to naïve oysters (Adlard and Nolan, 2015).

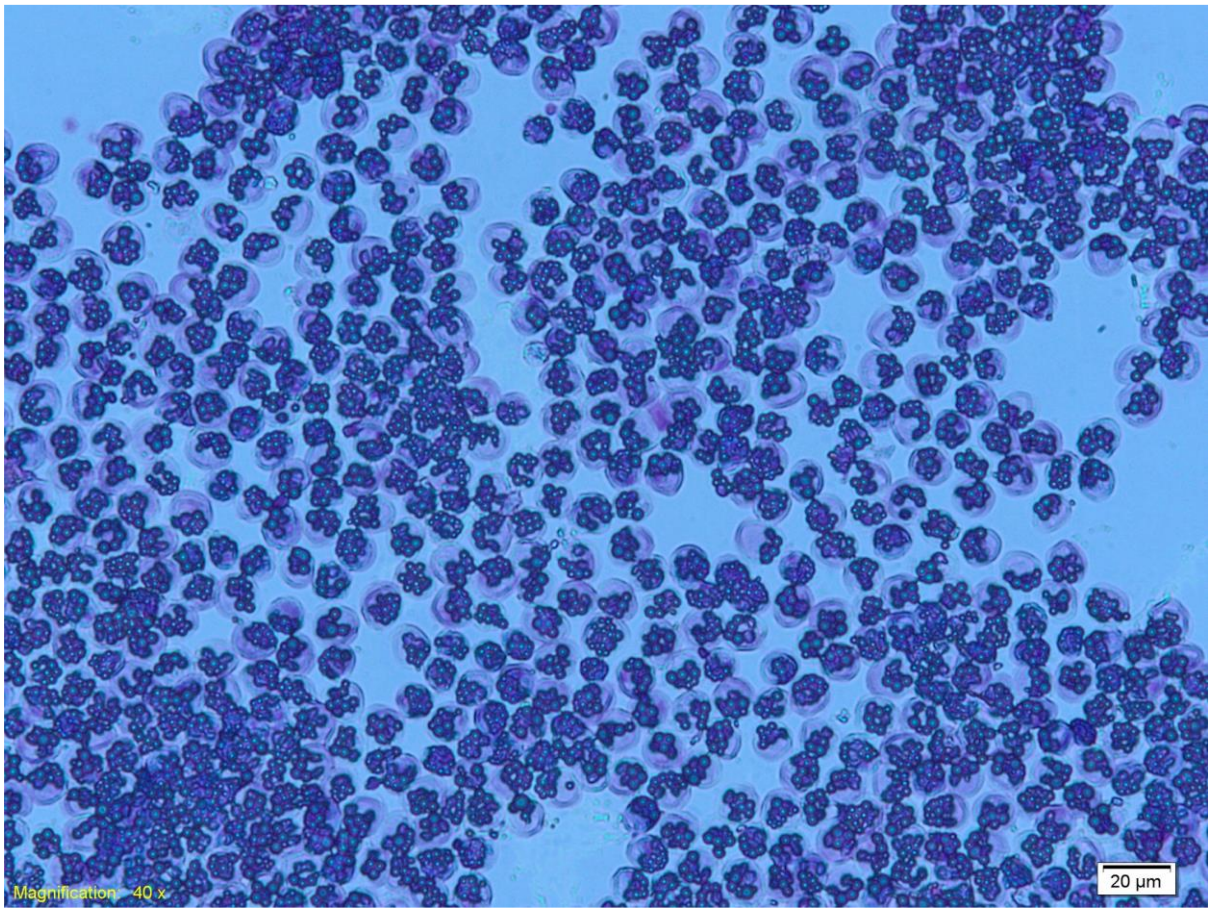

Figure 5: Purified *Marteilia sydneyi* sporonts, the causative agent of QX disease of *Saccrostrea glomerata*. Image is at 40x magnification. Image produced by Cheryl Jenkins and Jeffrey Go at the New South Wales Department of Primary Industries.

4.1.3 Management strategies of parasitic diseases

Attempts to reduce the impact of these parasitic diseases revolve around the development of breeding programs, modified husbandry practices, and quarantining affected areas (Nell et al., 2000; Smith et al., 2000; Ragone Calvo et al., 2003; Green et al., 2011; Lynch et al., 2014). Of these strategies, breeding for disease-resistance has been the most successful (Ragone Calvo et al., 2003; Dove et al., 2013a; Dove et al., 2013b; Lynch et al., 2014). Dual resistance has been bred into *C. virginica* against dermo and MSX disease, leading to an improved survivability of approximately 30-60% when compared to control oyster stocks (Ragone Calvo et al., 2003).

Similarly, a breeding programme carried out in Ireland since 1988 has successfully mitigated the damage of *B. ostreae* on *O. edulis* populations, culminating in an increased survival rate of 75% of market sized adult oysters, relative to 5-10% before the breeding programme began (Lynch et al., 2014). Breeding for disease-resistance has also been successful for *S. glomerata* against QX and winter mortality, with oyster mortality decreasing from 97% to 28% for QX, and 52% to 23% for winter mortality (Dove et al., 2013b). Modified husbandry practices are used to limit the exposure time of the oyster to the parasite, this can be done by altering the growing height of the oysters, or by transplanting oysters after the disease period has passed. Modified husbandry practices can be seen with winter mortality, in which *S. glomerata* are grown at a position located 15-30 cm higher in the tidal range than the typical growth height (approximately mid-tide level) (Smith et al., 2000).

4.2 Bacterial aetiological agents

4.2.1 Disease process of bacterial pathogens

Bacterial disease outbreaks are often sudden, resulting in severe mortality in a matter of days or weeks (Jeffries, 1982; Friedman and Hedrick, 1991; Bricelj et al., 1992). *Roseovarius crassostreae*, the aetiological agent of ROD in *C. virginica* causes sporadic outbreaks during the summer months, with mortalities up to 75% (Bricelj et al., 1992). For vibriosis of *C. gigas*, mortalities can exceed 90% within a period of only 24 hours (Takahashi et al., 2000). While *Nocardia crassostreae* the causative agent of *C. gigas* acts slower, resulting in mortalities up to 47% over 34 days (Friedman and Hedrick, 1991).

Lesions are common symptoms for oysters affected by ROD, nocardiosis, and vibriosis, and spat are often the most at risk for infection (Jeffries, 1982; Bricelj et al., 1992; Bower, 2006). In addition, *R. crassostreae* colonises the inner shell surface of *C. virginica*; the oyster responds to this intrusion through the formation of conchiolin (organic compound secretions involved in shell formation) deposits on the shell, which is thought to act as a barrier to contain further bacterial infection (Boardman et al., 2008). Additional pathological symptoms include lesions on the mantle, degradation of muscles and tissues, infiltration of hemocytes into the epithelium of the oyster, as well as lesions under the hinge ligament (Bricelj et al., 1992). Conchiolin deposits filled with bacteria and necrotic cells are also observed in vibriosis of *C. gigas* (Ralph et al., 1999). Conversely, conchiolin deposits aren't seen in nocardiosis, instead oysters display green pustules and lesions on a number of different oyster tissues (Bower, 2006).

A number of different *Vibrio* species cause disease in *C. gigas*, resulting in either vibriosis or bacillary necrosis (Jeffries, 1982; Sugumar et al., 1998; Waechter et al., 2002). A summary of the known *Vibrio* pathogens can be seen in Table 2. *C. gigas* larvae and spat are typically affected by *Vibrio* infections (Jeffries, 1982; Elston et al., 2008). Vibriosis in oyster larvae involves tissue necrosis (Figure 6) and abnormal swimming culminating in mortality (Jeffries, 1982). Vibriosis of spat can lead to lesions and necrosis of the tissues (Elston et al., 2008). As seawater temperatures rise with climate change, the spread and growth of bacteria such as *Vibrio,* which prefer warmer waters, has been predicted to be enhanced (Martinez-Urtaza et al., 2010; Vezzulli et al., 2016). Notably, an elevation in surface seawater temperature was linked to the resurgence of the oyster pathogen *Vibrio coralliilyticus* on the North American Pacific Coast, where it was responsible for a major *C. gigas* mortality event (Elston et al., 2008; Richards et al., 2015).

Table 2 *Vibrio* pathogens of *Crassostrea gigas* and their affected life stage. Bacterial pathogens are typically isolated from diseased oysters and used in virulence assays to determine pathogenicity.

Bacterial agent	**Stage affected**	**Reference**
V. tubiashii	Larvae	(Jeffries, 1982; Hada et al., 1984; Takahashi et al., 2000)
V. splendidus	Larvae	(Sugumar et al., 1998)
	Spat	(Waechter et al., 2002)
	Adult	(Garnier et al., 2007)
V. alginolyticus	Larvae	(Luna-González et al., 2002)
	Adult‡	(Go et al., 2017)
V. splendidus group	Spat	(Gay et al., 2004)
	Adult	(Garnier et al., 2007)
V. aestuarianus	Spat	(Saulnier et al., 2009; Saulnier et al., 2010)

	Adult	(Garnier et al., 2007; Saulnier et al., 2010)
V. lentus	Spat	(Saulnier et al., 2010)
V. harveyi	Spat	(Saulnier et al., 2010)
	Adult‡	(Go et al., 2017)
V. coralliilyticus	Spat	(Elston et al., 2008; Richards et al., 2015)
V. crassostreae	Spat†	(Lemire et al., 2015; Bruto et al., 2016)
	Adult‡	(Go et al., 2017)

368 †Based on supplementary information for the production of specific pathogen free (SPF)
369 oysters

370 ‡Used in an inoculation cocktail comprised of *V. alginolyticus*, *V. splendidus*, *V. harveyi* and
371 *V. crassostreae*

372

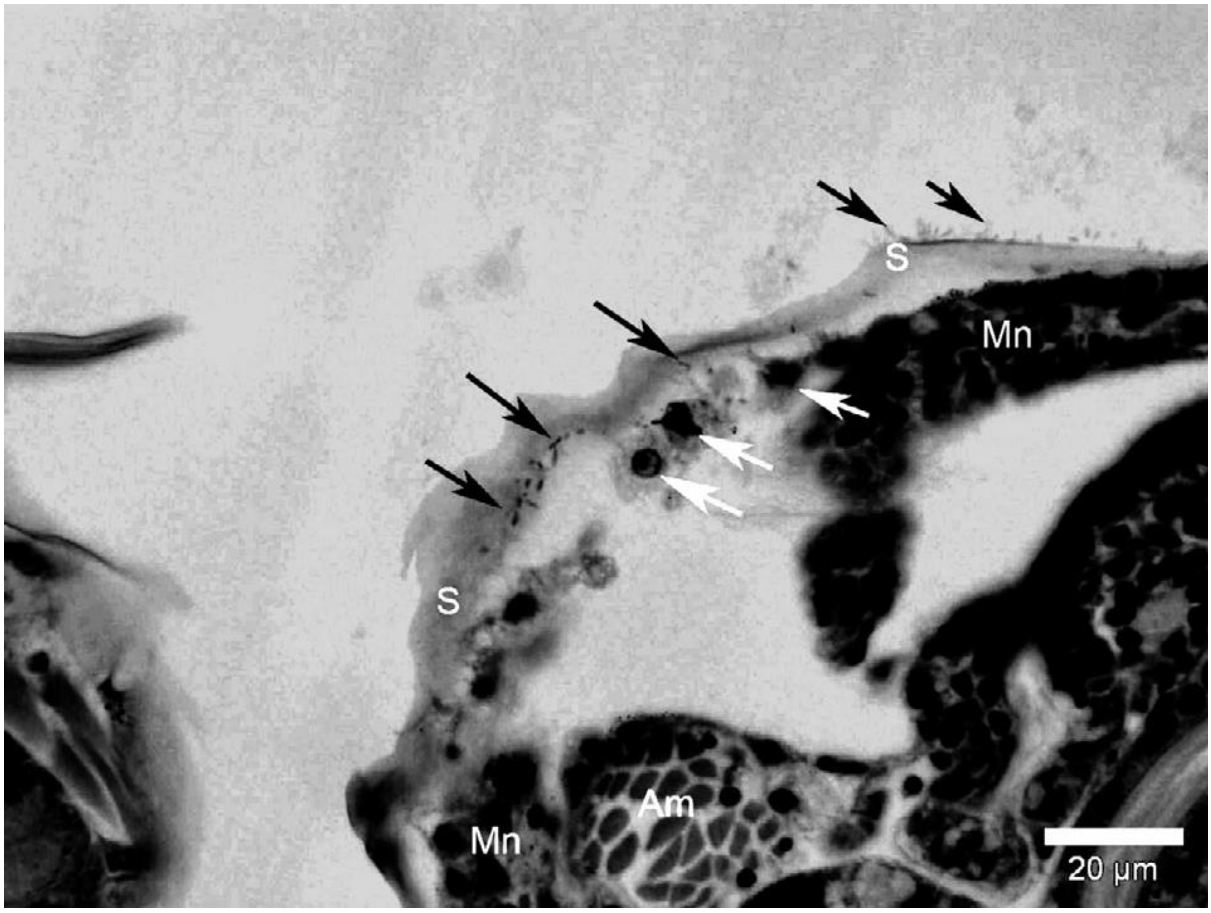

Figure 6: Histological section of *Crassotrea gigas* larvae, with a persistent *Vibrio* infection (black arrows), as well as necrotic epithelial cells (white arrows). Larvae tissue are marked as S (shell), Mn (mantle) and Am (adductor muscle) (Elston et al., 2008). Published by Diseases of Aquatic Organisms, © Inter-Research 2008.

While vibriosis tends to affect larvae and spat, experimental injections of adult oysters with *Vibrio* species, including *V. aestuarianus*, *V. splendidus*, *V. harveyi* and *V. crassostreae* (Garnier et al., 2007; Saulnier et al., 2010; Go et al., 2017) has also been shown to induce mortality, with a weakening of the adductor muscle and necrotic oyster tissues observed (Garnier et al., 2007). However, the injection of bacteria into oyster hemolymph/tissues may not be a good model for the natural transmission of *Vibrio* infections in the environment. Often *Vibrio* infections, particularly from the *V. splendidus* group, are found to occur concurrently with a herpesvirus infection (OsHV-1) (Segarra et al., 2010; Pernet et al., 2012; Keeling et al., 2014; de Lorgeril et al., 2018) with a recent study highlighting a synergistic, polymicrobial infection process, in which the oyster immune system is suppressed following OsHV-1 infection, allowing for bacteraemia to occur (de Lorgeril et al., 2018).

4.2.2 Environmental reservoirs and transmission of bacterial pathogens

Often, bacterial infections are opportunistic, requiring an environmental stressor or immune suppression of the oyster host before infection occurs (Bricelj et al., 1992; de Lorgeril et al., 2018). No studies have identified environmental reservoirs for *N. crassostreae* and *R. crassostreae*, while *Vibrio* species are ubiquitous in the environment and are commonly found in the water column, sediments, vegetation, and associated with other organisms (Vezzulli et al., 2010; Chase et al., 2015). Given the worldwide distribution of vibriosis, it is possible that *Vibrio* bacteria are members of the oyster microbiome that are awaiting favourable conditions to cause disease, such as with OsHV-1 infection (de Lorgeril et al., 2018) or with the acquisition of virulence plasmids through horizontal gene transfer (Bruto et al., 2016). Whereas *N. crassostreae* and *R. crassostreae* are localised to the USA northwest coast and USA east coast respectively (Friedman et al., 1991; Bricelj et al., 1992), because of this, there likely exists an unknown seasonal environmental reservoir for these pathogens.

Regarding transmission, laboratory transmission studies of ROD indicate that *R. crassostreae* is transmissible with symptoms arising 3 to 7 weeks after cohabitation with infected oysters (Lewis et al., 1996). Conversely, laboratory transmission of *N. crassostreae*, has not been successful (Friedman et al., 1991) suggesting either an unknown transmission element is required to infect new oysters, or that the infection is opportunistic, requiring environmental stressors such as the high temperatures typically seen during summer months, in order to induce disease (Friedman et al., 1991). Transmission of *Vibrio* species from infected to naïve oysters is likely bacterial species dependent. While one study was able to cause vibriosis in naïve animals by cohabiting them with oysters injected with a mixture of *V. splendidus* and *V. aestuarianus* (De Decker and Saulnier, 2011), another study was unable to transmit vibriosis when using a *Vibrio* cocktail made of *V. alginolyticus*, *V. splendidus*, *V. harveyi* and *V. crassostreae* (Go et al., 2017) possibly contrasting a difference in experimental methodology, or a difference between the transmission of different *Vibrio* species.

4.2.3 Management strategies for bacterial pathogens

No control measures are currently employed to contain nocardiosis of *C. gigas* or for ROD of *C. virginica*. Often vibrio blooms due to favourable environmental conditions (warm water and excess nutrients) are the cause of vibriosis for larvae and spat in hatchery settings (Elston et al., 2008). Monitoring environmental conditions and water quality may help predict *Vibrio*

outbreaks, possibly allowing farmers to change their water source in hatchery settings, or to remove oysters from the environment until the bloom has passed.

4.3 Viral aetiological agents

Of these economically valuable oyster species, only one virus, ostreid herpesvirus 1 (OsHV-1), has been identified as a major disease-causing pathogen (Hine et al., 1992; Friedman et al., 2005; Burge et al., 2006; Segarra et al., 2010; Jenkins et al., 2013; Lopez-Sanmartin et al., 2016; Mortensen et al., 2016). OsHV-1 primarily infects and induces mortality in *C. gigas* larvae and spat, as well as young adult oysters, with observed mortality rates ranging between 40 to 100% (Hine et al., 1992; Friedman et al., 2005; Segarra et al., 2010). OsHV-1 has been linked to a number of large mortality events across the globe and is continuing to spread (Burge et al., 2006; Segarra et al., 2010; Lopez-Sanmartin et al., 2016; Mortensen et al., 2016). Oysters infected with OsHV-1 display both lesions and cellular infections throughout the gills, mantle, digestive glands and in the hemocytes, whereby cells show altered cellular morphology, such as abnormal shapes, enlarged nuclei, nuclear fragmentation and nuclear inclusions (Hine et al., 1992; Renault et al., 1994; Friedman et al., 2005). OsHV-1 infected larvae have also been observed to have reduced feeding capacity and impaired swimming abilities (Hine et al., 1992; Renault et al., 2001)

Since its characterisation, a number of variant forms of OsHV-1 have been discovered (Arzul et al., 2001; Segarra et al., 2010; Martenot et al., 2011). Of these, a micro-variant form, named OsHV-1 µvar (Segarra et al., 2010), has been associated with mortality outbreaks in a number of countries (Segarra et al., 2010; Jenkins et al., 2013; Keeling et al., 2014; Mortensen et al., 2016). This micro-variant form has a number of nucleotide substitutions and deletions that distinguish it from the original variant (Segarra et al., 2010). Infection by OsHV-1 µvar acts to suppress the oyster's immune system thereby allowing opportunistic bacteria (such as *Vibrio* bacteria) to cause bacteraemia (de Lorgeril et al., 2018), and the oyster microbiome also shifts in response to viral infection (de Lorgeril et al., 2018). Furthermore, treating OsHV-1 µvar infected oysters with antibiotics significantly reduces the number of mortalities (Petton et al., 2015). As the oyster microbiome can act as a source of opportunistic pathogens (Lokmer and Wegner, 2015), further studies are required to examine the relationship (and possible interactions) between OsHV-1 µvar and the oyster microbiome.

OsHV-1 has been experimentally transferred to naïve oysters within the laboratory (Dégremont et al., 2013; Petton et al., 2015). Notably, it has also been demonstrated that OsHV-

1 resistant oysters infected with OsHV-1 are unable to transmit the virus to naïve oysters, and resistant oysters maintained an overall lower viral load than non-resistant oysters (Dégremont et al., 2013). Management strategies have been focused on movement controls (quarantining affected areas) and the production of genetic lines of oysters resistant to OsHV-1, that are able to reduce viral replication and more easily recover from viral infection (Segarra et al., 2014).

4.4 Unknown aetiological agents

In recent decades a phenomenon known as 'summer mortality' has heavily impacted the *C. gigas* aquaculture industry globally. These disease outbreaks have occurred all over the world including France (Garnier et al., 2007; Segarra et al., 2010), Australia (Jenkins et al., 2013; Go et al., 2017), the USA (Friedman et al., 2005), Germany (Watermann et al., 2008), Ireland (Malham et al., 2009), Japan (Mori, 1979) and in recent years Sweden and Norway (Mortensen et al., 2016). Summer mortality is marked by the loss of over 30% of oyster stocks (Soletchnik et al., 2005; Soletchnik et al., 2007) and in some instances has been observed to result in 100% mortality (Burge et al., 2007). Summer mortality has been responsible for catastrophic losses of *C. gigas* harvests since the 1960's (Mori, 1979), but the mechanisms involved and if a pathogen(s) is responsible remains largely unknown. A number of different factors have been implicated in these mortalities, including rising seawater temperatures, eutrophication, infections by *Vibrio* species and the herpesvirus OsHV-1, but often the cause appears to be multifactorial (Malham et al., 2009; Dégremont et al., 2013; Lemire et al., 2015; Petton et al., 2015), involving the interplay of multiple biotic and abiotic factors, which may affect the oyster immune system allowing opportunistic pathogens to take hold (Samain et al., 2007; Malham et al., 2009), and/or the abundance and virulence of pathogens. In this sense, summer mortality is an umbrella term that likely encompasses a number of different diseases with known or unknown aetiological agents. The bulk of recent research suggests a major role for OsHV-1 in summer mortality, with many research groups detecting this virus when disease outbreaks occur (Friedman et al., 2005; Burge et al., 2006; Burge et al., 2007; Segarra et al., 2010; Jenkins et al., 2013). It is notable however, that OsHV-1 was not detected in a recent summer mortality event in Australia (Go et al., 2017). Likely, periods of high temperature and low salinity acted to stress the oyster, resulting in immune suppression (Go et al., 2017), and allowing for bacterial infection to occur. This is evidenced with OsHV-1, in which infection acts to suppress the oyster's immune system allowing for bacteraemia to kill the host (de Lorgeril et al., 2018).

5.0 The role of the environment in facilitating disease outbreaks

The environment within which an organism resides, the pathogens to which it is exposed to, and the host's physiology (including the microbiome) can be considered an "interactome" that influences disease dynamics (Figure 7) (Arthur et al., 2017). The concept of the interactome is particularly relevant to oysters given that they filter large quantities of water, thereby increasing the chance of exposure to pathogens. However, while there has been a substantial amount of research into the mechanisms behind diseases of oysters due to the global economic importance of these species, only recently have studies taken a more holistic approach to unravelling the interactome (Pernet et al., 2016). As a result, there is a need to move beyond viewing oyster diseases from a classical perspective (Koch's postulates; one disease one pathogen), to a more ecological viewpoint of disease.

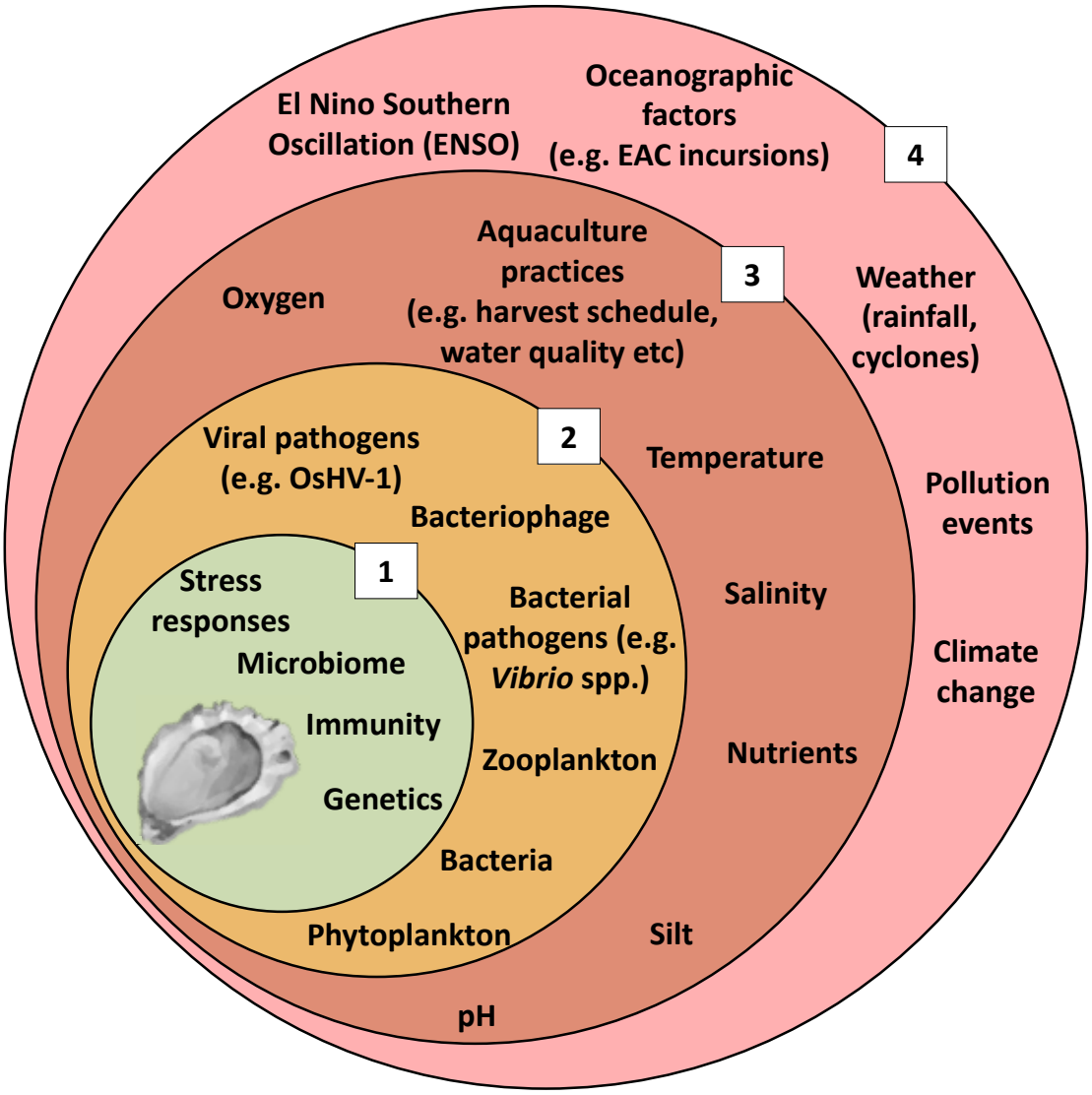

Figure 7: The interactome/synergism of oyster diseases. The outer rings are large scale environmental events (e.g. climate change) that influence the lower rings (e.g. temperature) allowing for a cascade effect that eventually influences microbial communities and pathogens (e.g. increased pathogen proliferation), that can then act on the oyster host.

There is growing evidence that environmental factors are critical in the spread and severity of oyster diseases (Ford, 1996; Petton et al., 2013; Mortensen et al., 2016). A summary of the environmental parameters that have been found to influence oyster diseases is presented in Table 3.

504 Table 3 Environmental factors that influence oyster diseases

Disease	Influential environmental parameters	References
Dermo	Increased winter temperature Increased salinity	(Burreson and Ragone Calvo, 1996; Ford, 1996; Cook et al., 1998; Soniat et al., 2012)
MSX	Increased winter temperature Increased salinity	(Haskin and Ford, 1982; Ford et al., 1999)
ROD	Increased temperature Increased salinity	(Lewis et al., 1996)
QX	Increased temperature Decreased salinity for spores	(Wesche et al., 1999)
Winter mortality	Dry autumns Increased salinity Decreased temperature	(Roughley, 1926; Butt et al., 2006; Nell and Perkins, 2006)
Marteiliosis	Increased temperature	(Berthe et al., 1998; Audemard et al., 2001)
Bonamiasis	Decreased temperature Increased salinity Higher pH‡	(Arzul et al., 2009)

Denman Island disease	Decreased temperature	(Hervio et al., 1996; Bower et al., 1997)
Nocardiosis	Increased temperature Lower dissolved oxygen	(Friedman et al., 1991; Engelsma et al., 2008)
Vibriosis	Higher temperature to increase *Vibrio* growth Low salinity inhibits *Vibrio* infectivity	(Lacoste et al., 2001; Elston et al., 2008; Richards et al., 2015)
OsHV-1	Increased temperature for viral replication Increased temperature for viral transmission Rainfall	(Jenkins et al., 2013; Petton et al., 2013; Renault et al., 2014)
Summer mortality	Chlorophyll *a* Temperature Turbidity Salinity Nutrients (Ammonium, Phosphate, Nitrate, Nitrite, Silicate)	(Soletchnik et al., 2007; Malham et al., 2009)

‡Observation made by the authors that more acidic media increased parasite mortalities.

5.1 Temperature

In marine environments, sea temperature is a major driver of oyster disease outbreaks with temperature shifts mostly dictated by the seasons, although oceanic phenomena (such as marine heat waves) can also play a role (Table 3). Warmer temperatures are known to affect the severity and prevalence of dermo, MSX, ROD, marteiliosis, QX, nocardiosis, vibriosis, OsHV-1 and summer mortality, while bonamiasis is most prominent during cooler water temperatures (Ford, 1996; Lewis et al., 1996; Wesche et al., 1999; Arzul et al., 2009; Malham et al., 2009;

513 Green et al., 2011; Petton et al., 2013). As a result, marteiliosis, nocardiosis, summer mortality
514 (including OsHV-1), MSX and ROD disease outbreaks occur, or are more severe, during the
515 summer months (Friedman et al., 1991; Berthe et al., 1998; Boettcher et al., 1999; Friedman et
516 al., 2005; Soletchnik et al., 2007; Engelsma et al., 2008; Watermann et al., 2008), with
517 outbreaks of vibriosis occurring during unusually warmer than normal summer temperatures
518 (Lacoste et al., 2001; Elston et al., 2008). Where cooler temperatures would normally supress
519 disease, there is evidence that unusually warm winters are a catalyst for increased intensity of
520 dermo and MSX outbreaks in the following summer (Burreson and Ragone Calvo, 1996; Ford,
521 1996; Cook et al., 1998; Ford et al., 1999). It's not always clear why warmer temperatures
522 induce disease outbreaks, but there is evidence that enhanced pathogen replication,
523 transmission, and stress to the host are likely determinants (Taylor, 1983; Gilad et al., 2003;
524 Lokmer and Wegner, 2015; Tout et al., 2015).

525 Laboratory- and field-based studies have identified clear temperature thresholds that facilitate
526 pathogen transmission. For the pathogens *R. crassostreae, M. refringens* and OsHV-1, the
527 highest levels of transmission occur at temperatures greater than 18 °C (Lewis et al., 1996), 17
528 °C, (Audemard et al., 2001) and 13.4 °C (Petton et al., 2013) respectively. In the field, disease
529 outbreaks by these pathogens occur at slightly elevated temperatures, exceeding 20 °C for ROD
530 and marteiliosis (Berthe et al., 1998; Boettcher et al., 1999), and 16 °C for OsHV-1 (Renault
531 et al., 2014), indicating that pathogen colonisation is only one aspect of disease causation and
532 that conditions that favour growth and increased host susceptibility also drive outbreaks.
533 Consistent with this, ROD disease onset is reduced from 7 weeks at the temperature permissible
534 temperature of 18 °C to only 3 weeks when the temperature is increased to 25.9 °C, following
535 transmission at 18 °C (Lewis et al., 1996). Regarding effects on the oyster host, warmer
536 temperatures of 21 °C are sufficient to reduce the numbers of hemocytes in the *C. gigas*
537 hemolymph, as well as reducing their phagocytic ability, as was demonstrated by oyster
538 hemocytes challenged with *V. anguillarum* (Malham et al., 2009).

539 In contrast to the examples above, some pathogens have greater impacts under cooler
540 temperatures. The viability of *M. sydneyi* spores is highest when temperature is reduced from
541 25 °C to 15 °C (Wesche et al., 1999), while *B. ostreae* shows improved survivability at 4 °C
542 compared to temperatures at 15 °C and above (Arzul et al., 2009). Furthermore, outbreaks of
543 winter mortality disease routinely occur in late winter or early spring (Roughley, 1926; Spiers
544 et al., 2014).

5.2 Salinity

Salinity shifts have been implicated as key factors in outbreaks of dermo, MSX, ROD, QX, bonamiasis, vibriosis and summer mortality. Each oyster species has an optimal salinity concentration for growth, with 15-18 ppt (parts per thousand), 20-25 ppt, 20 ppt and 25-35 ppt being the optimal range for *C. virginica*, *C. gigas*, *O. edulis* and *S. glomerata* respectively (Nell and Holliday, 1988; Wallace, 2001; FAO, 2016a; c). Shifts from these optimal ranges can occur following rainfall events, periods of extended drought, tidal changes and from wind-driven flow (Geyer, 1997; Drexler and Ewel, 2001; Schmidt and Luther, 2002; Da Costa et al., 2016). Infections from dermo routinely occur at salinities above 9 ppt, with the greatest infections occurring above 15 ppt (Burreson and Ragone Calvo, 1996), which is within the optimal range of growth for *C. virginica* (Wallace, 2001), although once an oyster is infected, the infection can persist under salinity levels as low as 1-13 ppt (Andrews and Hewatt, 1957). Long periods of minimal rainfall, also lead to an increase in dermo disease intensity and prevalence, which is thought to be related to increased salinity levels (Soniat et al., 2012).

For *P. marinus* (>15 ppt) and *H. nelsoni* (>15 ppt), infections occur within the optimal range of growth for their host (15-18 ppt for *C. virginica*). MSX disease severity is increased when the salinity is greater than 15 ppt, which is also within the optimal salinity range for *C. virginica* (Haskin and Ford, 1982). The protozoan, *B. ostreae* and the spores of *M. sydneyi* prefer high salinity (Wesche et al., 1999; Arzul et al., 2009). *M. sydneyi* spores showing heightened viability with increasing salinity, with an optimum viability at 34 ppt (Wesche et al., 1999) corresponding to the optimal salinity range of 25-35 ppt for *S. glomerata*. *B. ostreae* shows greatest survival in salinities greater than 35 ppt (Arzul et al., 2009), which is beyond the optimal salinity concentration (20 ppt) for *O. edulis*.

Salinity levels can also impact bacterial diseases such as ROD and vibriosis. Transmission of ROD readily occurs at salinities greater than 18 ppt, the upper limit for *C. virginica*, and while infections do occur at lower salinities (10 ppt and 14 ppt) mortality rates are significantly decreased (Lewis et al., 1996). Conversely, mortality from *V. coralliilyticus* and *V. tubiashii* infection in *C. virginica* decreased from 100% and 70.7% respectively to 0% by reducing the salinity levels from 28 ppt to 9.6 ppt (Richards et al., 2015). Rates of summer mortality are also correlated with low salinity, with oyster mortalities the greatest during the low autumn-winter salinity period (Soletchnik et al., 2007).

With the exception of *B. ostreae*, the salinity concentrations that allow for infections by the protozoans are within the optimal range for their host. While bacterial infection and mortality caused by *R. crassostreae* (>18 ppt), *V. coralliilyticus* (28 ppt) and *V. tubiashii* (28 ppt) all occur outside the hosts optimal salinity range (15-18 ppt) possibly indicating that bacteria require an external stressor to allow for disease progression to occur, while protozoan parasites do not.

5.3 Dissolved oxygen and pH

N. crassostreae induced mortalities are correlated with lower dissolved oxygen concentrations, possibly through an impact on the hosts ability to combat this pathogen (Engelsma et al., 2008). In addition, hypoxic environments have been shown to increase the acquisition and infection intensity of *P. marinus* infections in *C. virginica* (Breitburg et al., 2015; Keppel et al., 2015), while pH does not appear to play a role in *P. marinus* infection dynamics (Keppel et al., 2015). Decreased pH levels also significantly affect the formation and dissolution of the *C. virginica* shell, which can potentially increase oyster susceptibility to disease and predation (Waldbusser et al., 2011a; Waldbusser et al., 2011b). The combination of decreased pH and a hypoxic environment reduces the ability of hemocytes to create reactive oxygen species (Boyd and Burnett, 1999), which would ultimately hamper their ability to combat microbial infections. Previous studies have shown that acidification of water (<pH 5.5) from acid sulphate soil runoff can reduce *S. glomerata* growth, degenerate oyster tissues and lead to higher mortality rates (Dove and Sammut, 2007a; b). In contrast, another study observed no correlation between pH and *M. sydneyi* infection of *S. glomerata* (Anderson et al., 1994), possibly indicating that pH is more influential on the *S. glomerata* oyster host, rather than influencing the protozoan parasite itself. In addition, *S. glomerata* acclimated to acidic water through the incorporation of CO_2 into the oyster rearing tanks were shown to have a reduced tolerance to shifting salinity levels and temperature (Parker et al., 2017).

5.4 Nutrients

The possible role of nutrients in summer mortality disease outbreaks was first considered in the 1960's, when outbreaks of summer mortality in *C. gigas* occurred in the Matsushima Bay, Japan, a region subject to heavy eutrophication (Mori, 1979). However, since this initial evidence, the role of nutrients in oyster disease and mortality events has rarely been directly studied. Concentrations of phosphate, nitrate, nitrite, silicate and ammonium were elevated during *C. gigas* summer mortality outbreaks in Ireland and Wales, while in subsequent

laboratory experiments mortality of oysters from these environments was only induced following the additions of elevated nutrient concentrations (Malham et al., 2009). To our knowledge, this is the only study to examine the role of nutrients on oyster disease in depth. Although, a previous study has shown that growing oysters in nutrient enriched seawater led to mortality rates five times greater than those oysters in non-enriched seawater (Lipovsky, 1972). In a more general context, the role of nutrients, specifically from oyster feed, on oyster larval growth and survival has previously been reviewed (Marshall et al., 2010), with a general pattern of larvae diet strongly influencing larvae survival, as well as the need to supplement the larvae diet with protein as they progress through their life cycle (Marshall et al., 2010).

5.5 Translocation

While not an environmental factor, translocation is a common practice in the aquaculture industry and can unknowingly introduce pathogens to naïve areas. Examples of previous introductions of disease include marteiliosis and dermo (Alderman, 1979; Friedman and Perkins, 1994). Marteiliosis was spread from one affected area to other parts of France and then Spain, resulting in the introduction of *M. refringens* to these areas (Alderman, 1979). Dermo was historically located in the Chesapeake Bay, but persistent introductions of infected oysters to the north-eastern USA led to the establishment of dermo in these areas (Friedman and Perkins, 1994; Ford, 1996). Often though, translocation alone is not sufficient. Environmental conditions must be favourable to the pathogen to facilitate disease establishment and progression (Ford, 1996).

6.0 The relationship between the oyster microbiome and disease

Evidence for the importance of the microbiome has been building since the term "microbiome" was first coined in 1988 (Lisansky, 1988). Arguably, the bulk of the microbiome research has been focussed on humans, with specific compositions of the human gut microbiome correlated with a number of disorders/diseases (Turnbaugh et al., 2006; Abraham and Cho, 2009; Heijtz et al., 2011). In other organisms, the microbiome influences animal behaviour and their susceptibility to pathogens (Hosokawa et al., 2008; Koch and Schmid-Hempel, 2011), for example, the microbiome of *Drosophila melanogaster* (fruit fly) strongly drives the mating behaviour of this insect (Sharon et al., 2010). Using these examples, it is likely that the microbiome of oysters also plays a key role in oyster health, behaviour or through some contribution to the oyster disease process.

The role of the oyster microbiome in mortality outbreaks is an area of research yet to be fully explored. To date, previous research has shown that the microbiome can shift under a multitude of different stress treatments, such as translocation, starvation, temperature, infection and antibiotic stress (Green and Barnes, 2010; Wegner et al., 2013; Lokmer and Wegner, 2015; Lokmer et al., 2016a; Lokmer et al., 2016b). The microbiome also changes with different seasons (Pierce et al., 2016) and with translocation to laboratory conditions (Lokmer et al., 2016a). Additionally, while external abiotic factors can influence the microbiome, the within microbiome-interactions (between microbial organisms within a microbiome) can also play a role in bacterial community composition (Lokmer et al., 2016a) and destabilisation of this community can facilitate infection by *Vibrio* pathogens (Lokmer et al., 2016b) – this raises questions regarding the role of the oyster microbiome in disease resistance and susceptibility. Studies exploring the oyster microbiome during disease events are biased towards *C. gigas* and further towards summer mortality and the *Vibrio*-specific community.

The oyster microbiome is comprised of unique bacterial communities in each tissue, with the hemolymph bacterial community the most variable (King et al., 2012; Lokmer et al., 2016b). It has previously been proposed that destabilisation of the hemolymph microbiome can allow *Vibrio* bacteria to infiltrate the solid tissues causing a systemic infection (Lokmer et al., 2016b). There is increasing evidence that the microbiome of an organism plays an essential role in maintaining homeostasis (Shin et al., 2011; Earley et al., 2015). For instance, in humans the microbiome maintains immune homeostasis through reduction of inflammation (Kelly et al., 2004), provides host microbial defence (Fukuda et al., 2011), assists in nutrient degradation and uptake (Turnbaugh et al., 2009) and microbiome imbalances have been linked to chronic diseases such as Crohn's disease (Frank et al., 2007). The role of the microbiome in disease dynamics is emerging as an important factor in the progression and severity of oyster diseases (Petton et al., 2015). Reduced mortality in antibiotic-treated specific-pathogen-free (SPF) oysters subsequently exposed to OsHV-1 suggests an important role for the oysters microbiome in disease dynamics (Petton et al., 2015), in particular, the *Vibrio* community in healthy *C. gigas* harbours pathogens that can induce mortality in oyster larvae (Wendling et al., 2014). Furthermore, the non-virulent *Vibrio* portion of the oyster microbiome progressively shifts towards a virulent population during the onset of summer mortality while the remaining non-virulent *Vibrio* population appears to aid in causing the disease (Lemire et al., 2015). When virulent *Vibrio* strains are injected into oysters, the oyster microbiome does not become dominated by *Vibrio,* in fact, organisms from the genus *Arcobacter* become dominant (Lokmer

672 and Wegner, 2015). Similarly, by growing the *Vibrio*-injected oysters at higher temperatures
673 (22°C), the microbiome became more variable, with an increase in anaerobic bacteria,
674 including members of the *Clostridia*, which were found to be a particularly large component
675 of the microbial assemblage in dead oysters, possibly due to necrosis or anaerobic conditions
676 (Lokmer and Wegner, 2015). From the few studies focussed on examining the *C. gigas*
677 microbiome during a summer mortality disease outbreak, we can begin to make insights into
678 how the native microbial community can facilitate disease progression. *C. gigas* cultivated at
679 sites experiencing a summer mortality outbreak in Australia had a significantly different
680 microbiome structure than specimens from sites unaffected by summer mortality (King et al.,
681 2018) however, further research is required to determine the role of the whole microbiome in
682 disease dynamics. There is evidence that shifts in the *Vibrio* community can increase the
683 severity of disease, but it is unclear whether the whole microbial community, when stressed,
684 provides a protective role against disease, or aids in disease progression (Thurber et al., 2009;
685 Lemire et al., 2015; Tout et al., 2015).

686 To our knowledge, there has only been one study characterising the microbiome of *S.*
687 *glomerata* during a disease event, with evidence that infection by *M. sydneyi* reduces the
688 diversity of the oyster microbiome, with sequences with high homology to *Rickettsiale*-like
689 prokaryotes highly elevated in infected oysters (Green and Barnes, 2010). Changes in the
690 microbiome of *S. glomerata* in response to infection by *M. sydneyi* could further aid disease
691 progression but further studies are required to examine whether mortality can be reduced in
692 infected oysters with a more 'stable' microbiome.

693 The microbiome of *C. virginica* is understudied, particularly within the context of disease. To
694 date, the culture-able bacterial community has been studied in regards to its oil degradation
695 ability from the horizon oil spill in the Gulf of Mexico, with members of the *Pseudomonas*
696 genus as the dominant oil-degrading isolate (Thomas et al., 2014), and the microbiome of *C.*
697 *virginica* has been previously characterised using culture-independent techniques, in which the
698 oyster gut microbiome (intestinal contents) was found to more diverse than the stomach
699 microbiome, and the microbiome assemblage was influenced by spatial location (King et al.,
700 2012; Chauhan et al., 2014). A recent spatiotemporal study of the *C. virginica* microbiome
701 considered the influence of Dermo (Pierce et al., 2016). The *C. virginica* microbiome was
702 shown to change over seasons, with the microbial community composition significantly
703 influenced by water temperature, but the infection and severity of Dermo disease was not found
704 to be a significant determining factor of the microbiome (Pierce et al., 2016).

Similar to *S. glomerata* and *C. virginica,* studies of the *O. edulis* microbiome during disease events are lacking, indeed, studies characterising the healthy microbiome of *O. edulis* are also needed. To our knowledge, only one such study has examined the microbiome of *O. edulis,* with a focus on characterising the culture-able microbiome to examine shifts in the bacterial population over seasons, with isolates belonging to *Vibrio harveyi* dominant through the warmer months and *Vibrio splendidus* dominant during the colder months (Pujalte et al., 1999).

6.1 Oyster microbiome - future directions and challenges

Observational microbiome studies of *C. gigas* have begun to shed light on the dynamic interplay between the oyster microbiome, health, and disease. However, these studies are largely under-represented for *S. glomerata, C. virginica*, and *O. edulis*. It is becoming clear that applying stress to an oyster is sufficient to shift the oyster microbiome. This is seen with bacterial infection and temperature (Lokmer and Wegner, 2015), translocation (Lokmer et al., 2016b), starvation (Lokmer and Wegner, 2015), antibiotic stress (Lokmer et al., 2016a), exposure to a disease outbreak (King et al., 2018), and parasite infection (Green and Barnes, 2010). But it is not understood how the oyster microbiome responds before, during and after an environmental disease outbreak. Understanding this dynamic is crucial for determining the microbiome contribution to disease, and whether it can 'stabilise' following stress periods. However, carrying out environmental temporal studies are particularly challenging for a number of reasons: Firstly, in many cases the onset of disease can be very sudden and unpredictable. Secondly, holding/studying oysters in marine mesocosms (i.e. tanks or aquariums) significantly alters the oyster microbiome (Lokmer et al., 2016a) and will not be representative of an environmental outbreak. Thirdly, the oyster microbiome is highly heterogenous between replicate oysters (Lokmer et al., 2016a; King et al., 2018). Lastly, repeated hemolymph sampling of the same individual can cause local tissue infections resulting in an over-representation of bacteria assigned to the *Tenericutes* phylum (Lokmer et al., 2016a). To overcome these challenges, environmental temporal studies will need to have a high-resolution sampling regimen to capture the mortality event, likely coupled with a large number of biological replicates to overcome the heterogeneity in the oyster microbiome.

Breeding for disease resistance is a common aquaculture practice for the mitigation of oyster disease outbreaks (Dégremont, 2011; Dove et al., 2013b). Given the likely contribution of the oyster microbiome in oyster diseases (Lemire et al., 2015; Petton et al., 2015), there is a need to determine whether breeding for disease resistance also alters the oyster microbiome

composition and whether this alteration is, at least in part, responsible for disease resistance. If indeed the microbiome does play a role in disease resistance, another question is whether disease resistance oysters bred in one aquatic environment translate to another with different environmental parameters and likely microbiota? In the first instance, identifying whether disease resistance oysters have unique microbiomes will provide some insights into its protective role and stability after a disease event. Most importantly, characterising disease resistant oyster microbiomes may identify probiotic targets for the use in disease management strategies. However, as each tissue (including the hemolymph) has their own unique microbiome (Lokmer et al., 2016b), studies aiming to identify microbes unique to disease resistant oysters might need to homogenise the oyster or use a multi-tissue approach.

Moving beyond observational microbiome studies to manipulative experiments is another key challenge. Observational studies can provide insights into which microbes are driving shifts in the microbiome and be correlated to factors such as disease resistance, but do not provide information on the functional genes playing a role in the interactome. Metagenomics has emerged as a potential but expensive replacement for 16S rRNA microbiome sequencing (Handelsman, 2004). This technique provides both observational and functional data for microbiome analysis (Quince et al., 2017). However, as extracted DNA will contain a high ratio of eukaryotic to prokaryotic DNA, enrichment of prokaryotic DNA is required before sequencing (Thoendel et al., 2016).

Once the potential functional role of these microbes has been established, another key challenge is the cultivation and manipulation of specific members of the oyster microbiome. Cultivated organisms are required to characterise the interactions between these microbes (such as those correlated to disease resistance), the host, and pathogens (Bäumler and Sperandio, 2016), and to examine the probiotic effect of these microbes (Kapareiko et al., 2011). This may identify specific genetic elements that amplify or suppress oyster diseases, allowing for the development of monitoring programs to examine the abundance of these microbes/elements in commercial stocks and breeding programs.

7.0 Conclusions

Infectious diseases afflicting oysters have remained a constant barrier for the successful growth and sustainability of oyster aquaculture industries around the world. It is becoming increasingly apparent that the environment is an important factor driving the progression and severity of numerous oyster diseases and therefore, it is vital to consider how the environment can affect

pathogen invasion and host physiology when studying oyster diseases. Oysters exist in an ever-changing environment and are constantly exposed to new challenges. In fact, the history of oyster cultivation is riddled with attempts to overcome new and existing oyster diseases (René Robert and O'Mahoney, 2013). While the bulk of previous research has been focused on the presence of aetiological agents and their link to mortality outbreaks, future studies should begin to question why these mortality outbreaks happen, what stimulates them, and how can these mortality outbreaks be lessened by manipulating the conditions in which oysters are grown in. Furthermore, how does the microbiome fit into the disease process? Previous research has shown that the oyster microbiome can shift under a multitude of conditions, some of these conditions, such as infection stress, are able to completely replace commensal members of the microbiome with a more virulent community (Lemire et al., 2015), and microbiome destabilisation can facilitate pathogen spill over into different oyster tissues (Lokmer et al., 2016b). This virulent state can then amplify the severity of oyster diseases. Disruption of the *C. gigas* microbiome during summer mortality outbreaks is emerging as an important factor determining the progression and severity of this disease. Yet, microbiome research in other oyster species, and their role in disease, is lacking. As an oyster is exposed to a dynamic environment, the microbes they are exposed to will change, both over seasons (Wendling et al., 2014) and with climate change. Will a changing environment completely change the oyster microbiome? Will it result in more microbiome disruptions, allowing diseases to take hold more frequently? Or perhaps the oyster microbiome is more resilient than previously thought? Here we have begun to tease apart the interconnectedness of the external environment and oyster diseases, yet it is still unclear whether the external environment acts directly on the oyster physiology and microbiome, allowing pathogens to take hold, or whether it only regulates pathogen proliferation and infection, which will cause disease regardless of the state of the oyster and its microbiome state. Answering these questions will provide vital insights into the complexity of oyster diseases and in turn, will guide management practices of oyster aquaculture to reduce the economic impact of these debilitating oyster diseases.

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