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Mass mortality in bivalves and the intricate case of the Pacific oyster, Crassostrea gigas

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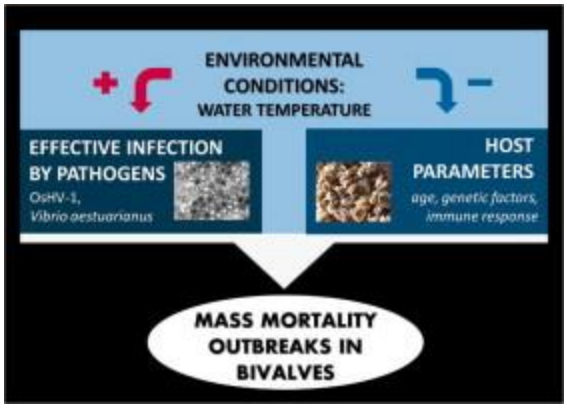
Abstract :

Massive mortality outbreaks in cultured bivalves have been reported worldwide and they have been associated with infection by a range of viral and bacterial pathogens. Due to their economic and social impact, these episodes constitute a particularly sensitive issue in Pacific oyster (Crassostrea gigas) production. Since 2008, mortality outbreaks affecting C. gigas have increased in terms of intensity and geographic distribution. Epidemiologic surveys have lead to the incrimination of pathogens, specifically OsHV-1 and bacteria of the Vibrio genus, in particular Vibrio aestuarianus. Pathogen diversity may partially account for the variability in the outcome of infections. Host factors (age, reproductive status…) including their genetic background that has an impact on host susceptibility towards infection, also play a role herein. Finally, environmental factors have significant effects on the pathogens themselves, on the host and on the host-pathogen interaction. Further knowledge on pathogen diversity, classification, and spread, may contribute towards a better understanding of this issue and potential ways to mitigate the impact of these outbreaks.

Graphical abstract :



Highlights

► Bivalve production and trade are harmed by recurrent mortality outbreaks. ► In *C. gigas*, outbreaks have increased (intensity and geographic area) since 2008.. ► Pathogens have been incriminated, particularly OsHV-1 and *Vibrio aestuarianus.* ► Host/pathogen and environmental factors affect the outcome of such infections. ► Mitigation of their impact requires additional data on pathogen diversity and spread..

Keywords : Pacific oyster, Spat, OsHV-1, Vibrio aestuarianus, Mortality

Valérie Barbosa Solomieu and Tristan Renault participated equally to this work and should be considered as primary co-authors

1 Introduction: overview of massive mortality outbreaks in bivalves related to viral and bacterial infections

1.1 Bivalve production and its vulnerability to pathogens

Aquaculture is an expanding sector with both economic and social relevance: in 2012, global aquaculture production attained an all-time high of 90.4 million tons (US$144.4 billion). Along with fisheries, aquaculture ensures the livelihoods of 10–12% of the world's population. The specific production of marine mollusks (oysters, mussels, clams, cockles, arkshells, and scallops) represents 13 million tons, with a value of US$13.8 billion (FAOSTAT, 2012). However, the production and the trade of marine bivalve species are vulnerable to the adverse impacts of environmental conditions and disease outbreaks. These outbreaks may affect all stages throughout the production process: larvae and post-larvae in hatcheries, as well as juvenile and adults, generally reared in an open environment. Massive mortalities may involve the complete loss of production stocks, with serious economic consequences. Increased hatchery production, livestock translocation, and species diversification contribute to increasing the occurrence and spread of infectious diseases. In recent years, disease outbreaks have significantly affected farmed oysters in Europe but also in other parts of the world (Paillard *et al*. 2004, FAO 2012).

1.2 Mortality outbreaks in bivalve production associated with bacterial diseases

Bacterial diseases, caused in particular by members of the genus *Vibrio* have been linked to mortalities in numerous bivalve species (Beaz-Hidalgo *et al.* 2010; Paillard *et al.* 2004). Bacillary necrosis, for example, initially described in larvae of *Mercenaria mercenaria* (Guillard 1959, Tubiash *et al.* 1965) touched by high mortality rates, also affects *Crassostrea virginica, Ostrea edulis, Argopecten irradians* and *Teredo navalis*. In addition to the species initially recognized as the causative agents of this necrosis: *V. alginolyticus, V. tubiashii* and *V.*

anguillarum (Tubiash *et al.,* 1970 and 1986), other pathogenic species have been recently identified. In the case of juveniles, four syndromes associated with bacterial detection have been reported to affect bivalves at this stage: Juvenile Oyster Disease (JOD) in *C. virginica* (Bricelj *et al.* 1992; Boettcher 1999, 2000), and, in the Pacific oyster, hinge ligament erosion disease (Dungan and Elston 1988; Dungan *et al.* 1989), chronic abscess syndrome (Elston *et al.* 1999) and spat mass mortality (Lacoste *et al.* 2001; Waechter *et al.* 2002).

Bacterial infections have however also been reported to result in mortalities in adult bivalves. Mass mortality outbreaks of clams (*Venerupis philippinarum*) leading to serious economic losses in France and Spain (Castro *et al.* 1990, 1992; Figueras *et al.* 1996; Paillard *et al.* 1989) have been associated with Brown Ring Disease (BRD), caused by *Vibrio tapetis*. Nocardiosishas also been linked with mass mortality episodes of adult Pacific oysters (*Crassostrea gigas*) in Japan (Takeuchi *et al.* 1955), California, Washington, and British Columbia (Elston 1993) and more recently the Netherlands (Eglesma *et al.* 2008). The etiological agent, *Nocardia crassostreae*, has also been shown to be pathogenic and lethal to flat oysters (*Ostrea edulis*) (Bower *et al.* 2005; Lauckner 1983).

Rickettsia-like organisms (RLOs) have been related to severe diseases and mortality outbreaks of marine bivalve molluscs including sea scallops (*Placopecten magellanicus)* (Gulka *et al.,* 1983 and 1984) and (*Pecten maximus*) (Le Gall *et al.* 1988), giant clams (*Hippopus hippopus* and *Tridacna gigas*) (Norton *et al.* 1993a and 1993b), clams (*Venerupis rhomboids*) (Villalba *et al.* 1999), pearl oysters (*Pinctada maximus and Pinctada fucata*) (Wu and Pan 1999a; Wu and Pan 1999b; Wu and Pan 1999c); oysters (*Crassostera ariakensis*) (Wu and Pan 2000; Sun and Wu 2004) and hard clams *Mercenaria mercenaria* (Meyers 1981). Other affections caused by bacterial agents but not necessarily associated with mortality events were reviewed in a recent paper (Romalde and Barja 2010).

1.3 Mortality outbreaks in bivalve production caused by viral infections

Viruses interpreted as members of various families (Papovaviridae, Togaviridae, Retroviridae, Reoviridae, Birnaviridae and Picornaviridae) have been described in bivalves (Farley *et al.* 1972; Farley 1976; Farley 1978, Meyers 1979; Oprandy *et al.* 1981; Rasmussen 1986; Bower 2001). Few studies have involved molecular identification and/or experimental *in vivo* trials confirming the affiliation and/or the pathogenicity of these agents (Renault and Novoa 2004). Infections by irido-like viruses were associated to massive mortality outbreaks of *Crassostrea angulata* between 1967 and 1973 (Comps *et al.* 1976; Comps and Bonami 1977; Comps and Duthoit 1979) and are interpreted as the main causes of the eradication of this species from European culture areas. The presence of herpes-like viruses has also been associated with disease outbreaks involving substantial mortalities in different bivalve mollusk species including Pacific oysters, flat oysters and European flat oysters (Farley *et al.* 1972; Hine *et al.* 1992; Comps and Cochennec 1993; Renault *et al.* 1994 a, b; Hine 1997, Hine and Thorne 1997).

Mass mortality outbreaks in association with the detection of a herpes-like virus were reported among larvae of hatchery-reared Pacific oyster for the first time in France during the summer of 1991 (Nicolas *et al.* 1992). At the same time, a herpes-like virus associated with larval mortality of hatchery reared Pacific oysters was reported in New Zealand (Hine *et al.* 1992). In the summer of 1992 and 1993, sporadic high mortalities (90-100%) occurred among batches of *Crassostrea gigas* larvae in several French hatcheries. Reduction in feeding and swimming activity of larvae was observed three to four days after fecundation. Significant mortality occurred by day 6, with 100 % mortality by days 8-10 in most batches. Transmission electron microscopy examination showed the presence of herpes-like virus particles in infected larvae (Renault *et al.* 1994). The pathogenic capacity of the virus was demonstrated through experimental trials conducted on axenic larvae inoculated with

suspensions obtained from infected larvae (Le Deuff *et al.* 1994). In New Zealand, mass mortalities (60% to 100%) occurred in Pacific oyster larvae in 1991 in a hatchery in Auckland. Larvae affected by this mortality episode were examined by transmission electron microscopy and appeared to be infected with a herpes-like virus (Hine *et al.* 1992).

In France, mass mortality outbreaks (80 to 90%) among 3-7 month-old Pacific oysters were first reported in July of 1993 along the French Atlantic coast (Renault *et al.* 1994). The presence of herpes-like virus particles in the connective tissue of gills and mantle from moribund spat was demonstrated through transmission electron microscopy (Renault *et al.* 1994). Thanks to capsid purification and molecular identification a new viral species infecting Pacific oyster larvae and spat was defined. This virus was called ostreid herpesvirus 1 (OsHV-1) (Le Deuff and Renault 1999; Davison *et al*. 2005)

A statistical correlation between OsHV-1 PCR positivity and mortality could be established in batches of oyster spat collected from French oyster rearing areas from 1992 to 1997 (Renault *et al.* 2000). Another epidemiological survey conducted from 1997 to 2006 by the French Repamo network (National Network for Surveillance and Monitoring of Mollusk Health) confirmed this association, with a peak in viral detection in May/June (Renault 2010; Garcia *et al.* 2011). In the United States, OsHV-1 was also detected by PCR in oyster spat affected by mass mortality episodes in California during the summer period (Friedman *et al.* 2005; Burge *et al.* 2006; Burge *et al.* 2007).

2 OsHV-1 infection and massive mortality outbreaks affecting *C. gigas* spat

2.1 Geographical distribution

Pacific oysters are produced worldwide: in Europe (France, Spain, United Kingdom, Ireland Channel islands, Norway), America (USA, Canada, Mexico, Argentina, Brazil, Chile,

Ecuador, Peru, the Falkland Islands), Asia (Singapore, China, Taiwan, Japan, Republic of Korea, Hong Kong), Africa (Morocco, Tunisia, South Africa, Namibia, Senegal) and Oceania (Australia, New Zealand, and New Caledonia) (FAO 2014). Not all of these production areas are concerned by massive mortality outbreaks.

Herpesviruses infecting bivalve molluscs are virulent pathogens of both larval and seed oysters. Collectively, these viruses have been described as herpes-like viruses or oyster herpesvirus (OsHV). Mortalities of juvenile oysters associated with detection of herpesviral DNA have been reported in France, New Zealand, Mexico, Spain, and the USA (Burge *et al.* 2006; Friedman *et al.* 2005; Hine *et al.* 1992; Renault *et al.* 1994; Renault *et al.* 2000 ; Vazquez-Juarez *et al.* 2006; Elandaloussi *et al*. 2009).

More recently, mass mortality episodes of oyster spat were associated with the detection of a newly described OsHV-1 variant, called µVar (Segarra *et al.* 2010). The latter has been reported in Europe: initially, in France (Martenot *et al*. 2010 and 2011, Segarra *et al*. 2010; Renault *et al*. 2012) and Ireland (Peeler *et al*. 2012) in 2008-2009, and subsequently in Great Britain, in particular in England, in the Kent area, in 2010 (EFSA 2010; Lynch *et al*. 2012). In Spain, OsHV-1 was detected for the first time by nested PCR (Renault *et al*. 2000b; Renault and Lipart 1998) in adult Pacific oysters collected in Catalonia in 2005 and originating from France (Elandaloussi *et al*. 2009; EFSA 2010). However more recent studies demonstrated the presence of a virus interpreted as the variant µVar (Segarra *et al*. 2010) in samples collected from 2008 to 2010 during high mortality events in several locations (Roque *et al*. 2012). In Italy, simultaneous detection of the "reference type" and a virus interpreted as the variant µVar in the absence of any pathological sign was achieved in juvenile oyster originating from France and collected the Marche region (Dundon *et al*.2011).

Outside of Europe, in the USA, OsHV-1 was detected by PCR in oyster spat affected by mass mortality episodes in California (Friedman *et al.* 2005; Burge *et al.* 2006; Burge *et al.* 2007).

In Australia, a recent study was conducted to assess the spatial distribution of OsHV-1 associated with *Crassostrea gigas* mortality in Woolooware Bay (New South Wales, Australia) (Paul-Pont *et al.* 2013). Briefly, in October 2011 the authors placed healthy sentinel Pacific oysters in three different locations at three different tidal levels. OsHV-1 associated mortalities were closely monitored over 7 months. In two of the sites, the outbreaks started in November 2011 and mortalities affected 100% of the spat in less than one week. Only adults displayed differential mortality patterns in function of the rearing height. In the third southernmost location, massive mortality of spat oysters occurred three months later, in February and adult mortality was similar at all rearing heights. The disease remained active until April 2012. In France, the experimental demonstration of virus pathogenicity has not been achieved with all incriminated virus specimens. Moreover, detection of herpesviral DNA has also been occasionally achieved without any associated mortality. For instance, in Japan, PCR detection of OsHV-1 DNA was achieved in wild and cultivated Pacific oysters (Moss *et al.* 2007; Renault *et al.* 2011; and 2012, Shimahara *et al.* 2012). The amplified sequences were similar but not identical to the variant µVar. However, the pathogenicity of the detected specimens has not been demonstrated (Shimahara *et al.* 2012). Sequence variation raises the issue of the genetic diversity of OsHV-1, which will be analysed in the following paragraph

2.2 Virus diversity

OsHV-1 is a DNA virus that was recently classified in the Malacoherpesviridae family from the Herpesvirales order (Davison *et al.* 2009). This virus has been associated with mortality outbreaks in the Pacific cupped oyster, *Crassostrea gigas*, since 1991. With the development of molecular tools, a variant, named OsHV-1var (Arzul *et al.* 2001a) was described. This "variant" presented several modifications in the "C region", which encompasses ORFs 114, 4 and 5, including a 2.8 kpb deletion (Arzul *et al.* 2001a) (Figure 3). It was associated with

mortality episodes affecting *Ruditapes phillipinarum* (Arzul *et al.*, 2001a), *C. gigas* and *Pecten maximus* (Arzul *et al.*, 2001b) in French hatcheries. Despite the above mentioned differences, the "reference type" and the variant var are considered representatives of a single viral species (Arzul *et al.*, 2001a).

Since 2008, a significant increase in the occurrence, intensity and geographic distribution of these outbreaks has been reported. This augmentation has been related to the detection of a particular OsHV-1 variant called µVar. This variant was described on the basis of specific polymorphisms in ORF4 (GenBank accession no. HQ842610-1) and ORFs 42-43 (Segarra *et al.* 2010). In particular, ORF4 of the variant µVar is affected by a deletion at a microsatellite site (12 consecutive nucleotides followed by a deletion of one adenine) that does not exist in the « reference » genome of OsHV-1 (GenBank accession no.AY509253) (Davison *et al.* 2005; Segarra *et al.* 2010).

The infectivity and strong pathogenicity of the variant µVar was shown by injection of oysters with affected spat homogenates (Schikorski *et al.* 2011a and b). Although µVar specimens have been detected in samples collected after 2008, a PCR assay targeting ORF4 yielded positive results in samples collected in France in 2004 and 2005 suggesting the presence of µVar or related variants prior to 2008 (Martenot *et al.* 2012). A summary of the detection of OsHV-1 and its variants in association with mortality events is provided in Figure 1.

Because the size of the deletion at the microsatellite site appears to be variable, additional variants have been defined based on this feature, such as OsHV-1 "µVar Δ9" and "µVar Δ15" (Martenot *et al.* 2011 and 2012). The variant "µVar Δ9" has been detected in moribund as well as in apparently healthy oysters (spat, juveniles and adults) collected during mortality outbreaks in France (2009 and 2010). The variant "µVar Δ15" was found in samples collected in France in 2010. It is currently unknown whereas these variants differ from the variant µVar

in terms of virulence. The term µVar should only be used for specimens displaying all the mutations described by Segarra *et al*. (2010) in ORF4 and ORFs 42-43.

The variations occurring at ORF4 have raised interest in the characterization of the genetic variability among OsHV-1 specimens. Recent studies have shown that the analysis of the sequences of three particular regions (ORF4, ORFs35-36-37-38 and ORFs42-43), both independently and as concatemerized units, make it possible to define different sub-groups within known «genotypes» (Renault *et al.* 2012). In this study, the described methodology was applied to the analysis of a set of 72 samples collected between 1993 and 2010 mainly in France but also in Ireland, USA, China, Japan and New Zealand. Briefly, results from French samples are as follows: samples collected between 1993 and 2003 were mostly (88%) identical to the reference type, one third (32%) of all samples collected between 2005 and 2008 displayed differences as they did not yield amplicons with the Del 36-37F2/Del 36-37R primer set and 47% samples collected in 2008 were identical to OsHV-1 µVar. In addition, the reported data suggest that OsHV-1 µVar and the reference type share a common ancestor the former is not directly derived from the latter. Finally, ORF4 appeared as the most polymorphic genome area distinguishing several genogroups.

Since the publication of these results, samples from a wide range of locations have been screened using the same approach. The generated data tend to indicate that the genetic diversity of the virus may be larger than anticipated and that the sole presence of the characteristic deletion at ORF4 may not be sufficient to classify the specimen as the variant µVar (Barbosa-Solomieu *et al.*, pers. comm.) as previously reported by Segarra *et al.* (2010). Further analysis of the genetic diversity of OsHV-1 may provide insights about the phylogenic relationships between specimens collected in different areas and the spread of the virus

2.3 Virus infection and environmental factors

2.3.1 Seawater temperature

Although OsHV-1 infections related to mortality events including the 2008-2012 outbreaks across Europe have mainly occurred in spring/summer months suggesting a key role of the water temperature, few studies have targeted the role of temperature on viral infection under experimental conditions (Le Deuff *et al.* 1994; Le Deuff *et al.* 1996; Sauvage *et al.* 2009). Le Deuff *et al.* (1996) showed the existence of a relationship between high water temperatures and both the production of viral particles and the mortality of Pacific oyster larvae. A study by Sauvage *et al.* (2009) suggests that, in laboratory conditions, a mean seawater temperature of 23.8°C and its rapid increase (2.3°C over 2 days) are favorable to the onset of an OsHV-1 outbreak among *C. gigas* spat. More recently, experimental assays demonstrated that successful infections of Pacific oyster spat by OsHV-1 can be achieved at water temperatures between 20-22°C (Schikorski *et al.* 2011a and b).

Several studies have identified seawater temperature as a key factor in terms of OsHV-1 detection during mortality outbreaks in the field. An OsHV-1 survey carried out in France from 1997 to 2006 showed that virus detection followed a gradient of increasing seawater temperatures along French coasts (Garcia *et al.* 2011). These authors also reported that the onset of mortality events was usually preceded by a rapid increase of mean seawater day temperatures In the USA, a similar data have been generated as seawater temperatures of 24-25°C have been recorded prior disease outbreaks (Burge *et al.* 2006; Burge *et al.* 2007). A study carried out in the Marennes-Oleron area (Charente Maritime, France) (Renault *et al.* 2014) also suggested that a rapid increase of seawater temperature beyond a particular threshold is a crucial trigger for mortality outbreaks related to OsHV-1 infection. Clegg *et al.* (2014) studied *C. gigas* mortality events that occurred in Ireland during the summer of 2011 and indicated an increase of mortalities along with seawater temperature until a peak was reached. This is similar to the observations made by Pernet *et al* (2014) in the Thau lagoon in France.

As a conclusion, high seawater temperatures appear as a main factor inducing OsHV-1 infection. Comparable observations have been made for herpesviruses infecting vertebrates: for example, temperatures between 18°C and 28°C have been shown to favor the onset and the severity of Koi herpesvirus infection in fish (Gilad *et al.* 2003; Hara *et al.* 2006; Pokorova *et al.* 2005; Saint-Hilaire *et al.* 2005; Sano *et al.* 2004; Yuasa *et al.* 2008). However, it remains difficult to define a precise temperature threshold leading to enhanced OsHV-1 expression or mortality. The temperature threshold was variable following the rearing site, ranging from 22°C to 25°C on the west coast of the USA (Burge *et al.* 2006; Burge *et al.* 2007) and from 16°C to 24°C in France (Pernet *et al*. 2014; Petton *et al.* 2013; Samain and McCombie 2008, Soletchnik *et al.* 1999). Moreover, Renault *et al.* (2014) suggested that viral contamination can occur among oysters in absence of mass mortality and when the water temperature is below 16°C.

2.3.2 Other environmental parameters

Other parameters known to influence mechanism of disease transmission in aquatic environments (hydro-dynamics, physical disturbances, host density/distribution, and variations of environmental parameters) have been suggested as factors influencing OsHV-1 infection (Barbosa-Solomieu *et al.* 2005; Burge *et al.* 2007; Friedman *et al.* 2005; Garcia *et al.* 2011, Paul-Pont *et al*. 2013; Renault *et al.* 1994a and b). Paul-Pont *et al.* (2013) assessed the spatial distribution of OsHV-1-associated mortality in Woolooware Bay (New South Wales, Australia) using healthy sentinel Pacific oysters and showed that mortality could depend on oyster position in the seawater column. They also suggested that OsHV-1 may be carried through water by particles, possibly plankton. Pernet *et al.* (2014) carried out a spatial and temporal study of a mass mortality outbreak related to OsHV-1 infection. They investigated oyster spat mortality in relation to energetic reserves and food quality in animals deployed at several locations in the Thau lagoon on the Mediterranean coast (France). These

authors showed that the dynamics of spat mortality was significantly correlated to differences in energetic condition, partly driven by variation in food quality and relative contribution of diatoms to the diet of oysters. Future studies should aim at developing improved methods to assess oyster mortality and follow stocks over time to better determine the influence of environmental factors on mortality.

2.4 Virus infection and host factors

2.4.1 Susceptible host species

Pacific oyster, *C. gigas*, Portuguese oyster, *C. angulata*, suminoe oyster, *C. ariakensis*, European flat oyster, *Ostrea edulis*, Manila clam, *Venerupis philippinarum*, carpet shell clam, *V. decussatus*, scallops, *P. maximus* are naturally infected (Arzul *et al.* 2001; Renault *et al.* 2001). Interspecies transmission from infected axenic larvae of *C. gigas* to axenic larvae of *C. ariakensis* and *O. edulis* was demonstrated under experimental conditions (Arzul *et al.* 2001b). An OsHV-1 suspension prepared from *V. philippinarum* infected larvae was able to infect *C. gigas* larvae, and a virus suspension from *C. gigas* was shown to infect *C. angulata* larvae (Arzul *et al.* 2001b).

OsHV-1 (µVar) DNA has been recently detected in France in blue mussel, *Mytilus edulis,* and in *Donax trunculus* by PCR and PCR product sequencing (Renault *et al.*, pers. comm.). However, it's no clear if those bivalves are disease susceptible or act as vectors.

2.4.2 Persistent infection

Apparently healthy oysters, including adults, may be PCR-positive for OsHV-1 DNA detection (Arzul *et al.* 2002; Moss *et al.* 2007, Sauvage *et al.* 2009, Segarra *et al.* 2014). Pépin *et al.* (2008) showed that DNA copy numbers mg^{-1} were high (up to 10^7) in oysters from populations with abnormal mortalities and low (lowest number detected 10^1) in populations with no abnormal mortality.

Mortality related to OsHV-1 detection is significantly lower or absent in adult oysters compared with other age groups (Arzul *et al.* 2002; Peeler *et al.* 2012). However, the virus (DNA, protein or particles) has been detected in tissues of adult oysters, including gonads (Arzul *et al.* 2002, Lipart and Renault 2002). These observations suggest that adults may be a source of infection for the most susceptible age groups, larvae or spat, particularly if the parent animals are under stress, e.g. from high temperature (Le Deuff e*t al.* 1996). However, it is not certain whether true vertical transmission (transmission within the gametes) occurs or whether transmission is strictly horizontal (Barbosa-Solomieu *et al.* 2005).

2.4.3 Susceptibility to the viral infection and viral immune response

Several studies support the hypothesis of a genetic basis underlying resistance to OsHV-1 infection in the Pacific oyster**,** *C. gigas* (Dégremont 2011, Sauvage *et al.* 2009, Segarra *et al.* 2014). In addition, differences in terms of susceptibility to the viral infection appeared associated with differences in host gene expression (Segarra *et al.* 2014). Sauvage *et al.* (2009) investigated the disease susceptibility of different families of Pacific oyster studying 3 groups of animals from 3 families in laboratory conditions. Significant differences in terms of mortality rates and OsHV-1 DNA detection were reported between oysters belonging to the different families. All animals were maintained in a single raceway and equally exposed to the risk of infection. Therefore, the obtained results strongly suggest the existence of differential susceptibility towards viral infection among oysters (Sauvage *et al.* 2009). Conversely, Segarra *et al.* (2014) reported a divergent response of Pacific oyster families in terms of mortality during an experimental OsHV-1 (µVar) infection. They also suggested that oysters are genetically diverse in terms of susceptibility to OsHV-1 infection. An initial phase of active virus replication characterized by an increase in the detected amounts of viral DNA and RNA, was followed by a rapid decrease in viral DNA and RNA detection. These results suggest that the

host immune defence was activated and that some oysters were able to circumvent OsHV-1 infection (Segarra *et al.* 2014).

Renault *et al.* (2011) are the first authors to report data on genes related to antiviral immunity in the Pacific oyster. These authors studied virus-induced genes in Pacific oyster haemocytes challenged by OsHV-1 through Suppressive Subtraction Hybridisation (SSH). Different genes already known as immune-related genes were thus identified. Investigation of oyster gene expression by real-time RT PCR showed a significant increase of MyD88 gene transcripts in experimentally infected oysters. Segarra *et al.* (2014) reported similar results and suggested that the over expression of MyD88 could be interpreted more as a marker of the infectious process and viral replication than a marker of an effective antiviral response. Recently, Green *et al.* (2014) carried out a study to determine how Pacific oyster ontogeny interacts with water temperature to influence the antiviral response against OsHV-1 infection. For this purpose, they measured the effect of temperature (12 vs 22 °C) on oyster immune response after poly I:C injection. They reported that the expression of genes related to immunity was influenced by temperature and oyster age (Green *et al.*, 2014).

2.4.4 Stress and viral infection

Stress conditions (handling, transport, crowding, modification of feeding, pesticides) have also been suggested as factors involved in the development of viral disease (Renault *et al.* 2010; Barbosa-Solomieu *et al.* 2005; Burge *et al.* 2007; Friedman *et al.* 2005; Garcia *et al.* 2011, Normand *et al*. 2014). Moreau *et al.* (2015) reported that exposure of Pacific oysters to a mixture of 14 pesticides was related to increased mortality rates that could be interpreted as an increased susceptibility to OsHV-1 infection.

2.5 Co-detection of OsHV-1 and vibrios

The co-detection of OsHV-1 and different *Vibrio* species including *V. splendidus* and *V. aestuarianus* has been reported during mass mortality events affecting Pacific oysters in Europe since 2008 (Efsa 2010). Saulnier *et al.* (2010) reported results obtained during a 4-year bacteriological survey (2003-2007) of French Pacific oysters. Bacteria identified as belonging to *V. Splendidus* clade and *V. aestuarianus* species were mainly detected in samples collected during mortality events. A large number of *V. harveyi* related strains were reported in association to 2007 oyster mortality outbreaks (Saulnier *et al.* 2010). OsHV-1 (reference type) DNA was also detected during this period in France in association with mortality events affecting Pacific oysters (Garcia *et al.* 2011). Keeling *et al.* (2014) conducted a field study during the 2010−11 mortality outbreak in New Zealand and detected OsHV-1 through a PCR approach. They also identified several Vibrio species: *V. splendidus, V. aestuarianus and V. alginolyticus*. These bacterial species have previously been associated with mortality in marine bivalves (Le Roux *et al.* 2002; Gómez-León *et al.* 2005; Saulnier *et al.* 2010). Identification of key Vibrio species in addition to OsHV-1 suggests that complex interactions may occur between different pathogens in the marine environment

3. Infection with *Vibrio aestuarianus* and massive mortality outbreaks affecting adult Pacific oysters

3.1 Geographical distribution

Vibrio aestuarianus was initially isolated and described by Tison & Seidler (1983) from seawater, oysters, clams and crabs from the Oregon and Washington coasts (USA), in absence of animal mortality. Epidemiological studies on *C. gigas* juvenile and adult mortality outbreaks reported along the French coast have revealed the presence of *V. aestuarianus* in moribund animals and their environment since 2001 (Garnier *et al.* 2007; Saulnier *et al.* 2010

Azandégbé *et al.* 2010). These French isolates, pathogenic for *Crassostrea gigas,* were included in a newly defined subspecies: *V. aestuarianus* subsp. *francensis* in opposition with the American type strain: *V. aestuarianus* subsp. *aestuarianus* (Garnier *et al.* 2008) (Figure 2). Since 2012, the frequency at which *V. aestuarianus* has been reported during oyster mortality outbreaks in France has increased (Garcia *et al*. 2014), suggesting a (re)emergence of this pathogen. However, by combining genome analyses and experimental infections on a large collection of strains, the authors did not observe any correlation between lethal dose, genotype and isolation date, suggesting that the emergence of a new virulent clonal strain is unlikely (Goudenège *et al.* 2014). Therefore, the recent outbreaks in France (Garcia *et al*. 2014; Goudenège *et al.* 2014) may be at least partially explained by a number of physiological disorders of oysters leading to an increased susceptibility to *V. aestuarianus,* and/or environmental factors (abiotic and biotic) favouring the multiplication, persistence or virulence of the bacteria.

Additionally, since 2001, *V. aestuarianus* has been detected in different sites in Europe in the absence of noticeable mollusc mortalities. Few environmental studies have reported its presence in coastal areas: Baltic sea (Eiler 2006), Spain (Montes *et al.* 2003), Hong Kong (Wang *et al.* 2006). More recently, an intensive field survey conducted as part of the European project Bivalife allowed its detection and isolation within the context of spat mortality in Italy (Goro lagoon) (Vezzulli *et al.* 2014, Domenghetti *et al.* 2014). However, the virus OsHV-1 was suspected to be implicated in those mortality events. Viral DNA was detected in high amounts in moribund animals and the *V. aestuarianus* strains that had been isolated were classified as non-virulent following experimental infections (Goudenège *et al.* 2014). Three *V. aestuarianus* strains were also isolated in absence of mortality in Spain (Galicia), from oyster, plankton and mussels (Romero *et al.* 2014). The classification of those isolates into either of the two subspecies is however still unknown.

3.2 Bacterial diversity

An initial study comparing 11 *V. aestuarianus* isolates from moribund oyster hemolymph revealed a very low diversity on the basis of 16S rRNA, *gyrB* and *toxR* genes, indicating a high degree of homogeneity (Garnier *et al.* 2008). Similarly, genomic analyses of 14 *V. aestuarianus* isolates revealed the cohesive genotypic structure of *V. aestuarianus* with relatively little diversity among genomes (Goudenège *et al.* 2014). This study confirmed that *V. aestuarianus* species grouped with two species containing fish pathogens, *V. ordalii* and *V. anguillarum*, into the Anguillarum clade (Goudenège *et al.* 2014), as previously suggested (Sawabe *et al.* 2013). Moreover, genome analyses revealed the existence of two virulent lineages with very low intra-clade and inter-clade diversity and containing each a majority of virulent strains (Goudenège *et al.* 2014). The question of the ecological signification of those groups has now to be addressed.

These studies are nevertheless mainly based on isolates collected during mortality events. The few non virulent strains that have been studied (isolated from zooplankton in Italy, cockles in France and healthy oysters in Spain) were found to be more diverse (Goudenège *et al.* 2014) suggesting that the diversity of environmental isolates is higher than the one of strains capable of causing oyster disease.

3.3 Bacterial infection and environmental factors (temperature and salinity)

Mortality reports analysed by the French surveillance network (Repamo) revealed that individuals affected by *V. aestuarianus* were preferentially detected in summer (Garcia *et al.* 2014), suggesting a link between pathogenesis with temperature, even if mortality were noticed all year-long. A first study revealed that juveniles and adult oysters are more sensitive to *V. aestuarianus* infection (Dégremont *et al.* 2014). However, the evolution of oyster

sensitivity to *V. aestuarianus* infection with age and/or size is currently unknown.

Temperature and salinity can influence virulence expression (i.e. pathogenesis) but also bacterial persistence and growth in the environment (Vezzulli 2013).

3.3.1 Effect of temperature and salinity on the virulence of *V. aestuarianus*

In experimental conditions, after intramuscular injection of cultured *V. aestuarianus,* mortality can be induced from 10°C to 32°C and extended from 24 hours (32°C) to 25 days (10°C) (Travers *et al,.* pers. comm.; Dégremont *et al.* 2014). Field studies indicated that temperatures between 19 and 23°C seem to favour bacterial growth and infection (Garnier *et al.* 2007). However, mortalities were also reported at lower temperatures (Dégremont *et al.* 2014).

Even if the demonstration of a direct impact of temperature and/or salinity on the expression of virulence genes is not available yet, those parameters surely have an indirect impact on bacterial virulence. One known virulence factor, the metalloprotease Vam (Labreuche *et al.* 2010) is regulated by quorum sensing mechanisms (De Decker *et al.* 2013), themselves controlled by bacterial growth (Waters and Bassler 2005). Under laboratory conditions, optimal conditions for *V. aestuarianus* growth ranged from 20°C to 25°C (Vezzulli *et al.* 2014) and the optimal salinity was around 20‰, with a generation time around 60 min at 20°C.

However, as demonstrated for mussels (Asplund *et al.* 2014), the impact of environmental factors can be mainly reflected on the host-pathogen interaction rather than the pathogen or the host taken individually.

3.3.2 Effect of temperature ad salinity on the persistence and growth of *V. aestuarianus*

Little information is available on the ecology of virulent strains of *V. aestuarianus* in the environment and the impact of environmental conditions on the persistence and niche colonization of the bacteria.

The first study on its ecology demonstrated that, regardless of salinity and temperature (test conditions ranged from 5 to 25°C and 20 to 35‰), *V. aestuarianus* can only persist a few days in seawater in the absence of nutrients (Vezzulli *et al.* 2014). So, despite the impact of temperature and salinity on bacterial growth (Garnier *et al.* 2008), *V. aestuarianus* presents a low persistence potential in seawater in the absence of nutrients or potential planktonic partner. This is in agreement with ecological studies that only sporadically detected its presence in seawater, without any correlation with temperature and salinity (Vezzulli *et al.* 2014, Domeneghetti *et al.* 2014).

Temperature and salinity can also influence the mode of life of the bacteria that can enter into a VBNC (Viable But Non Culturable) state to persist during winter (Azandégbé 2010). This author reported that small bacteria (<0.2 µm) with an intact membrane structure can be observed in seawater after a long term incubation at 5°C and 20‰. Similarly, in sediment, despite low temperatures (5°C), around 30% of the bacteria added in the mesocosm remained viable throughout the entire duration of the experiment and never dropped below 10^4 cells.ml^{-1} (Vezzulli *et al.* 2014).

In conclusion, in addition to having an impact on the pathogenesis of *V. aestuarianus*, environmental conditions appear to drive bacterial status, persistence and, potentially, niches. Conditions for bacterial persistence in environment and virulence expression are well described but we still need to define conditions where initial contamination / infection do not occur.

3.4 Bacterial infection and physiological factors

In co-infection experiments mixing *V. splendidus* LGP32 and *V. aestuarianus* 02/041 carried out at different dates on similar *C. gigas* batches, De Decker *et al.* (2011) proposed a link between reproductive status and survival performances of the oysters. They suggested that

reproductive effort leads to a state of physiological weakness resulting in an increased Vibrio susceptibility during summer. During reproductive season, oyster hemocytes were reported to display lower phagocytic activity and adhesion capacity. These immune functions are known to be targeted by the bacteria (Labreuche *et al.* 2006a-b), and particularly the secreted metalloprotease Vam (Labreuche *et al.* 2010).

The production of divergent Pacific oyster lines for reproductive allocation and/or the comparison of lineages with contrasted survival capacities could be a way to demonstrate correlations between reproduction and susceptibility to Vibrios.

4. Concluding remarks

Although infectious diseases appear as the main causes of mass mortality events in bivalve mollusks, as illustrated by the case of the Pacific oysters, environmental factors (water temperature, salinity, presence of pollutants, trophic conditions) may have an impact on the host and the pathogen themselves but also on the host/pathogen relationship.

Host factors, some of which are genetically driven also play a crucial role, as they have an influence on host susceptibility towards infection by viral and bacterial agents. In the presence of pathogens, the combination of a few or all of these parameters may result in mortality outbreaks in locations and under conditions that are seemingly unrelated. Further experimental trials mimicking particular field conditions may allow to better understanding host-pathogen interactions and the variability of the outcome of viral, bacterial and combined infections.

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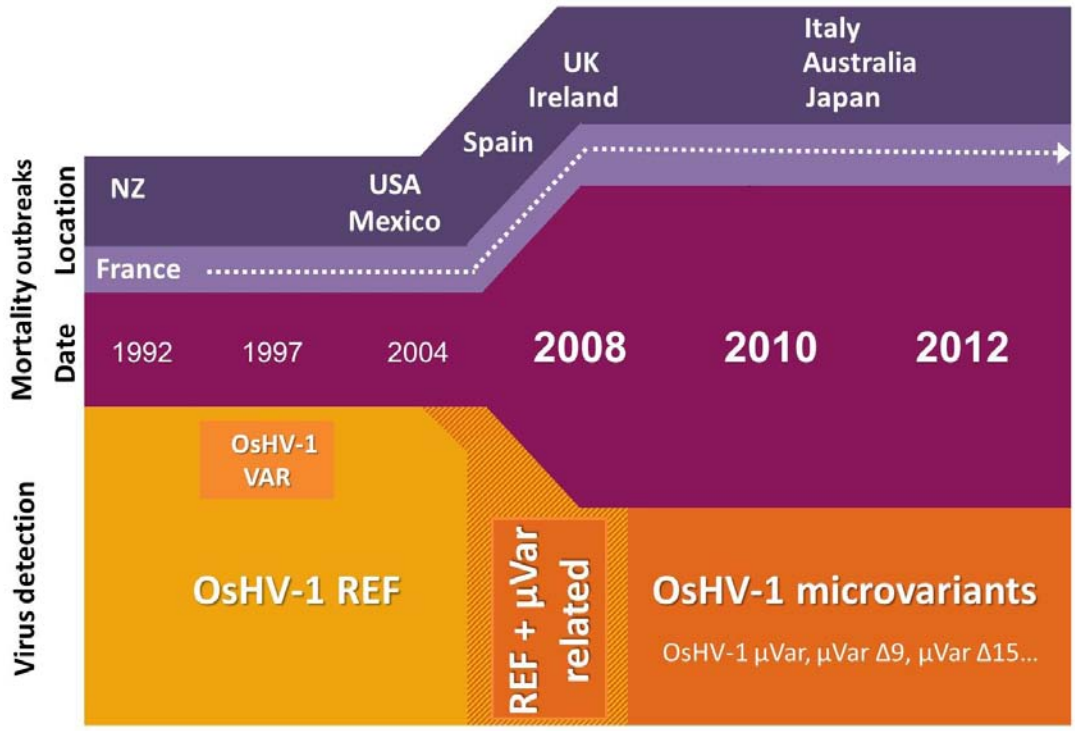
FIGURE LEGENDS

Figure 1 - OsHV-1 and its variants in association with mortality in bivalve molluscs

Figure 2 - First reports of isolation of *Vibrio aestuarianus* in the USA (Tison and Seidler 1983), Hong Kong (Wang *et al.* 2006), France (Garnier *et al.* 2007and 2008, Saulnier *et al.* 2010, Azebengbé *et al.* 2010), Spain (Romero *et al.* 2014 and Goudenège *et al*., under review), Italie (Vezzulli *et al*., 2014 and Goudenège *et al*.,2014), New Zealand (Keeling *et al*. 2014), Ireland (Cheslett *et al*. 2014).

Figure 3 – Location of the main target areas for the primary analysis of the genetic diversity of OsHV-1: "C region", ORF 4 and the C2/C6 primer set (adapted from Arzul *et al*., 2001 and Davison *et al*., 2005).



First reports of isolation of *V. aestuarianus*