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A previously undescribed ostreid herpes virus 1 (OsHV-1) genotype detected in the pacific oyster, *Crassostrea gigas*, in Ireland

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SUMMARY

Significant mortalities of the Pacific oyster, *Crassostrea gigas*, have been reported worldwide since the 1950s. The impact these re-occurring mortality events have had on the *C. gigas* industry has highlighted the necessity to determine the factors that may be causing these mortalities. This study investigated the possible role of ostreid herpes virus (OsHV-1) in *C. gigas* mortalities over 2 successive summers at 2 study areas in Ireland. A single sample of adult *C. gigas*, which had been experiencing mortalities at one of the sites was screened. Successive cohorts of *C. gigas* spat obtained from a hatchery outside Ireland was relayed to both sites in 2003 and in 2004. Spat were screened each year prior to relaying. Samples were collected every 2 weeks and mortality counts were recorded and observed at both sites. Polymerase chain reaction (PCR) analysis and subsequent sequencing indicated that a previously undocumented variant genotype of OsHV-1 was present in the single cohort of adult *C. gigas* and in seed and juveniles at both sites, in both years. Analysis suggests that the Irish OsHV-1 μvar variant genotype is closely related to OsHV-1 μvar, first described in France in 2008.

Key words: ostreid herpes virus-1, variant, *Crassostrea gigas*, mortalities, Ireland.

INTRODUCTION

Significant mortalities in *Crassostrea gigas* were first recorded along the Japanese Pacific coast in 1945 (Koganezawa, 1974) and in the 1950s in North America (Glude, 1975). The phenomenon intensified throughout the 1960s and early 1970s in North America, particularly in Washington, and in oyster culture areas in Japan (Woelke, 1961; Pereyra, 1964; Imai *et al.* 1965; Mori *et al.* 1965; Lipovsky and Chew, 1972; Koganezawa, 1974; Glude, 1975; Beattie *et al.* 1980). More recently, significant mortalities have been recorded from a number of European countries, in particular France (Comps and Cochennec, 1993; Renault *et al.* 1994, 2000*a*; Goulletquer *et al.* 1998; Soletchnik *et al.* 1999; Samain *et al.* 2004; Dégremont *et al.* 2005, 2007) and Ireland (Cotter *et al.*, 2010; European Food Safety Authority, 2010 (www.marine.ie/home/aboutus/newsroom/news/UpdateonOysterMortalities.htm). The main cause of 'summer mortality syndrome' has not been definitively determined; however, many factors including pathogens, elevated temperatures, physiological stress associated with gonadal maturation, aquacultural practices, water quality, predators, phytoplankton blooms and genetic factors are all known to contribute to *C. gigas* summer mortalities (Renault *et al.* 1994; Goulletquer *et al.* 1998; Berthelin *et al.* 2000; Lacoste *et al.* 2001; Le Roux *et al.* 2002; Dégremont *et al.* 2003; Huvet *et al.* 2004; Malham *et al.* 2009; Cotter *et al.*, 2010). A multi-factorial aetiology has been suggested (Dégremont *et al.* 2005; Samain *et al.* 2007; Soletchnik *et al.* 2007; Samain and McCombie, 2008; Cotter *et al.* 2010). Previous studies have investigated the role of ostreid herpesvirus 1 (OsHV-1) and variants as causative agents in *C. gigas* summer mortalities and have found this virus to be particularly associated with larval and juvenile mortalities (Renault *et al.* 1994, 2000*a*; Le Deuff and Renault, 1999; Arzul *et al.* 2002; Vásquez-Yeomans *et al.* 2004; Friedman *et al.* 2005; Burge *et al.* 2006, 2007).

Ostreid herpesvirus 1 (OsHV-1) belongs to the genus *Ostreavirus*, family *Malacoherpesviridae* which is included in the order *Herpesvirales* (Davison *et al.* 2009). Viruses belonging to the order *Herpesvirales* are relatively large double-stranded DNA (dsDNA) viruses and can cause latent and/or lytic re-occurring infections (Sandri-Goldin, 2006; Mettenleiter and Sobrino, 2008). OsHV-1 μvar is a variant of OsHV-1 and is considered to be a more virulent strain (European Food Safety Authority, 2010). OsHV-1 μvar was originally associated with *C. gigas* mortalities in France in 2008 and in 2009 (Repamo, 2009; Segarra *et al.* 2010) and has subsequently been observed in Ireland (http://web.oie.int/wahis/public.php?page=single_report&pop=1&reportid=8503), the March region in Italy

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(Dundon *et al*. 2011), the Netherlands (http://web.oie.int/wahis/public.php?page=single_report&pop=1&reportid=10749), the UK (http://web.oie.int/wahis/public.php?page=single_report&pop=1&reportid=9527) and south east Australia (http://web.oie.int/wahis/public.php?page=single_report&pop=1&reportid=10136). Other variants have been observed in a wide range of bivalves including oysters, scallops, clams and abalone (Hine and Thorn, 1997; Hine *et al*. 1998; Renault *et al*. 2000*b*; Arzul *et al*. 2001*a*,*b*; Renault and Arzul, 2001; Chang *et al*. 2005; Davison *et al*. 2005; Hooper *et al*. 2007; Moss *et al*. 2007; Tan *et al*. 2008; Samain and McCombie, 2008).

Pépin *et al*. (2008) reported that abnormal mortalities in oyster samples were associated with a viral load range of 6×10^3 to $5{\cdot}3 \times 10^7$ copies/μl or mg. There are an estimated 10^{30} viruses in the worlds' oceans and previously unknown viruses are still routinely discovered in well-studied organisms such as the panaeid shrimp (Flegel, 2006). In general, mortalities associated with viruses in the marine ecosystem are expected to arise sporadically when conditions become less favourable for the host, such as a physiological stress induced by significant increases in temperature, causing the host to become immunocompromised and unable to suppress an infection (Suttle, 2007). In the case of OsHV-1 μvar, such mortality events, which occur in an intensively cultured commercial bivalve, have become more regular and widespread.

The main objective of this study was to investigate the role of ostreid herpesvirus 1 in summer mortalities of adult and *C. gigas* seed and juveniles in the Irish Sea. This study was part of a larger study that looked at a range of biological and physical factors contributing to oyster mortalities along the coast of Ireland (Malham *et al*. 2009; Cotter *et al*. 2010).

MATERIALS AND METHODS

Study sites

Two field sampling areas were selected on the south east coast of Ireland: Dungarvan Harbour, Co. Waterford (52°03′N 007°35′W) where minimal mortalities had occurred and Bannow Bay, Co. Wexford (52°27′N 006°47′W) where more significant mortalities had previously been reported in Pacific oysters (Clarke, 1996). Bannow Bay, where *C. gigas* have been cultured since 1984, has an area of *ca*.1050 ha, is 8 km long, and between 1 and 3 km wide. The sea enters this shallow bay through a narrow inlet at the southern end. Little flushing occurs at low tide and water stays in the bay for 2 tidal cycles. Approximately 75% of the bay is exposed at low tide. Dungarvan Harbour is an open bay of *ca*. 1300 ha. On a national basis, it holds the largest standing stock of *C. gigas* and is the most productive area of operation in Ireland. Two sites were selected within each of the two study areas (Bannow Site A (BA), Bannow Site B (BB); Dungarvan Site A (DA) and Dungarvan Site B (DB)). These sites were located at corresponding shore heights, i.e. 4 h exposure on spring tides. BA is a muddy site where mortalities had previously occurred, BB, DA and DB are all sandy sites where minimal mortalities had been recorded. Sites BA, DA and DB were all accessible at low neap tide but spring tides were required to access BB.

Oysters

A sample of 29 market-sized *C. gigas* ($n = 29$) (24 months old, 60 + g) from Bannow Bay were provided by fishermen from stock that had been experiencing mortality in May 2003. In June 2003, a single cohort of hatchery-reared *C. gigas* spat (shell length ~ 15mm) was obtained from a hatchery outside of Ireland. An initial sample of 60 of these oysters was removed for screening by polymerase chain reaction (PCR) for OsHV-1 prior to relaying oysters at both Dungarvan and Bannow Bay (Dungarvan Bay A (DA), Dungarvan Bay B (DB), Bannow Bay A (BA), and Bannow Bay B (BB)). Oysters were randomly distributed into $0{\cdot}5$ m^2, 4 mm mesh bags on trestles at 2 densities used during the growing cycle: 1000 oysters bag^{-1} ($n = 5$ per site) and 600 oysters bag^{-1} ($n = 3$ per site). In June 2004, another single cohort of oysters was obtained from the same hatchery and an initial sample of 60 oysters was screened again prior to oysters being relayed to the same 4 sites, but at 1 density only (1000 oysters bag^{-1}).

In both 2003 and 2004, 60 oysters were sampled from July to October at 2-week intervals from each of the sites with the exception of BB. This site was accessible only on spring tides, and was generally sampled on a monthly basis.

Mortality

A total of 200 individuals were removed randomly from each bag every 2 weeks and were counted *in situ* to estimate oyster mortality. The percentage mortality was determined after counting the cupped empty shell only. Empty oyster shells were discarded after each count was completed. Sixty *C. gigas* were removed for PCR analysis while the remaining living *C. gigas* were returned to the bags.

DNA extraction and PCR

In the 29 adult oysters sampled in May 2003, 100 μl of haemolymph were withdrawn from the adductor muscle sinus of each oyster and stored at −20 °C. Initially phenol/chloroform extraction of DNA was carried out. However, poor template yield led to

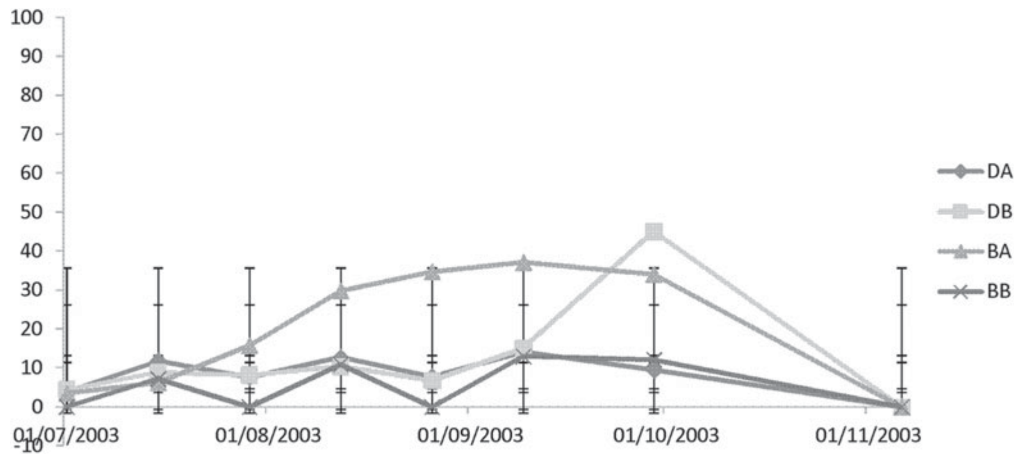

Fig. 1. Mean mortality (%) in *Crassostrea gigas* at Irish sites in 2003.

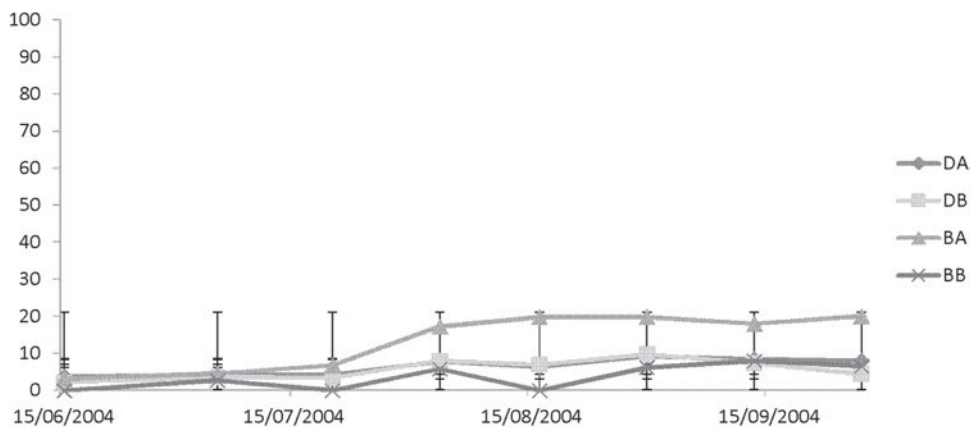

Fig. 2. Mean mortality (%) in *Crassostrea gigas* at Irish sites in 2004.

difficulties with direct sequencing, which resulted in subsequent DNA being extracted from the frozen haemolymph using 10% chelex resin (Sigma-Aldrich) (Walsh *et al*. 1991; Lynch *et al*. 2010). Gill samples ($n = 60$) were collected from the initial spat samples in 2003 and 2004 and on each subsequent sampling date and frozen at −20 °C. Initially, DNA was extracted using the phenol/chloroform extraction method (Winnepenninckx *et al*. 1993); however, similar issues with the template yield led to difficulties with direct sequencing. DNA was extracted from the corresponding paraffin-embedded tissue of a subsample of individuals (Dungarvan Bay ($n = 32$), Bannow Bay ($n = 31$)) which had all amplified products in the original PCR. Deparaffinization of the tissue was carried out (Shi *et al*. 2002) and DNA extractions were undertaken using protein precipitate and cell lysis using the QIAamp DNA Mini Kit (Qiagen™).

Screening for OsHV-1 was carried out using the C2/C6 primers and PCR conditions of Renault and Arzul (2001) using a Promega dNTP mix and a Thermo Hybaid PCR express thermal cycler. This PCR identifies oyster herpes DNA (709 bp expected product size) in region C, which encodes for 2 proteins of unknown function. As this region of the genome has proven to be variable and diagnostic in previous studies of OsHV-1 type viruses (Arzul *et al*. 2001*a*, *b*; Segarra *et al*. 2010) the present study focused on this region. Both negative and positive controls were included in each PCR, viral OsHV-1 DNA samples from heavily infected *C. gigas* prepared in 100 ng/l in TE buffer were used as positive controls, and deionized distilled water was used as a negative control.

Amplified PCR products from the chelex-extracted adult haemolymph and both spat subsamples were excised from the gel and products were pooled to increase the DNA concentration, 3–4 oysters approximately per pool keeping sites and adult and spat samples separate, and DNA was isolated and purified using the QiaQuick gel extraction kit (Qiagen™) prior to direct sequencing (MWG eurofins).

Construction of phylogenetic tree

Ten pooled PCR products from 33 individuals: 3 pooled samples representing 11 oysters from Dungarvan Harbour seed and juveniles, 3 pooled

Table 1. Results (% positive) from cPCR screening of *Crassostrea gigas* samples ($n = 60$) from Bannow Bay and Dungrvan Bay in 2003

Sample date	DA	DB	BA	BB
02/07/03	71	0	—	—
29/07/03	—	0	—	—
30/07/03	—	—	25	—
12/08/03	—	2	—	—
13/08/03	—	—	70	30
09/09/03	5	28	—	—
29/9/03	16	5	—	—
06/11/03	2	2	—	—
07/11/03	—	—	3	53

— Sample not screened.

Table 2. Results (% positive) from cPCR screening of *Crassostrea gigas* samples ($n = 60$) from Bannow Bay and Dungrvan Bay in 2004

Sample date	DA	DB	BA	BB
20/07/04	0	0	—	–
21/07/04	—	—	0	0
03/08/04	0	5	—	—
04/08/04	—	—	0	10
30/08/04	0	0	—	—
31/08/04	—	—	0	0
27/09/04	23	82	0	0

— Sample not screened.

samples representing 10 oysters from Bannow Bay seed and juveniles and 4 pooled samples representing 12 individuals from the adult oysters, were directly sequenced. Six sequences (forward and reverse) were obtained for each of the Dungarvan Harbour and Bannow Bay seed and juveniles and 8 sequences (forward and reverse) were obtained from the adult *C. gigas* at Bannow Bay. Individual sequences were manually trimmed to have the same sequence length. The forward and reverse sequence reads were aligned using the ClustalW algorithm (Geneious v.5.1.3, www.geneious.com, using the default options). The consensus sequence, based only on sequence for which both strands were present, of each alignment was matched against the National Centre for Biotechnology Information (NCBI) nucleotide database with BLASTn (Basic Local Alignment Search Tool) to confirm that the sequences were similar from the OsHV-1 genome. Alignments were edited to count each putative indel as one mutational tranversion (transition/transversion ratio of 0·1288) and the phylogenetic relationship among sequence variants was visualised using a Maximum Likelihood tree, with 1000 bootstraps, based on the Kimura 2 parameter model (Kimura, 1980) distances using the software package MEGA v.5.05 (Tamura *et al*. 2011).

RESULTS

Mortality

The percentage mortality was not quantified in the adult market-sized oysters but the oyster farmers had reported approximately 10% mortalities in the stocks at the time of sampling. During summer 2003, percentage mortality in the seed and juveniles varied from 14·2% in DA to 45% in DB. There was greater mortality in the higher density bags (1000 oysters bag-1) early in the season; however, there was no significant effect of density on mortality at the end of the sampling period (ANOVA $P = 0{\cdot}06$) (Fig. 1). Mortality levels were much lower in 2004 varying from 8% at BB to 19·8% in BA (Fig. 2).

PCR analysis

In the adult *C. gigas* haemolymph samples, products were originally amplified in 7% (2/29) of the oysters screened. In the PCR re-screening of the Chelex extracted adult *C. gigas,* DNA products of the expected size (709 bp) were amplified in 90% (26/29) of the sample.

The initial samples of *C. gigas* spat taken prior to relaying to both Irish locations in 2003 and 2004 were negative for OsHV-1 when screened by PCR. 900 and 960 individual seed and juvenile *C. gigas* were screened by PCR in 2003 and 2004 respectively (Tables 1 and 2). In 2003, products were amplified in 13 of the 15 sample periods (87%) screened. Products were initially observed in DA at 2 weeks (71% prevalence) and at all sites by 8 weeks post-relaying. Peaks in prevalence varied between sites at different times from a peak of 28% at DB in September to a peak of 71% at DA in July (Table 1). In 2004, products were amplified in 4 of the 16 samples (25%) screened. In 2004, it was 8 weeks post-relaying prior to positive results being obtained by PCR (DB and BB) and positive results were initially obtained in DA at 16 weeks post-relaying. No positive results were obtained from BA. The highest prevalence of OsHV-1 was observed in September 2004 at 16 weeks post-relaying at DB (82%) and at DA (23%). At BB, a single sample amplified products (10%) in August, 8 weeks post-relaying (Table 2).

In the PCR re-screening of the subsample of Dungarvan Harbour and Bannow Bay seed and juveniles in which DNA was extracted from paraffin-embedded tissue, products were visualized in 34% (11/32) of the Dungarvan Harbour sample and 32% (10/31) of the Bannow Bay sample.

Sequencing confirmed that the DNA sequences obtained from the adult oysters and the seed and juveniles at the two sites in Ireland were identical. In the BLASTn analysis, the Irish sequences (GenBank Accession number JQ963169) were similar to OsHV-1 (GenBank Accession number AY509253). Further, sequences were aligned and genetic variation was detected in the C region in the Irish OsHV-1 μvar sequences compared to all previously published

Fig. 3. Aligned DNA sequences of the C region amplified by the C2/C6 primer pair. Sequences were edited to show identical sequence length. Note the indels at 20 and 32 bp when comparing the French and Irish Sequences. Sequences are coded as follows; GenBank Accession number AY509253, Davison *et al*. 2005); OsHV-1 var (Arzul *et al*. 2001*a*, *b*); OsHV-1 μvar (Segarra *et al*. 2010); and Irish variant genotype (GenBank Accession number JQ963169).

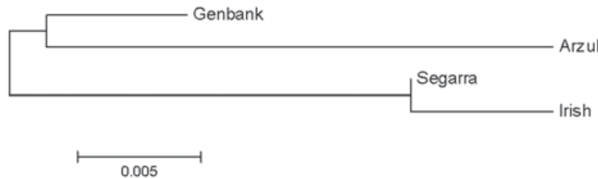

Fig. 4. Maximum likelihood tree showing the phylogentic (Kimura 2 parameter model distances) relationship among four OsHV-1 sequence variants, using all available variability. Sequences are coded as follows; GenBank, (GenBank Accession number Y509253, Davison *et al*. 2005); Arzul, (Arzul *et al*. 2000 *a*, *b*); Segarra, (Segarra *et al*. 2010); and Irish variant genotype (GenBank Accession number JQ963169).

sequences (i.e. GenBank Accession number AY509253, Arzul *et al*. 2001*a*,*b*; Segarra *et al*. 2010, Fig. 3). The Irish sequence was almost identical to the OsHV-1 μvar strain recently identified in France (Segarra *et al*. 2010) and now epizootic in Europe. The differences consisted of 1 nucleotide deletion (adenine/thymine) and 1 nucleotide insertion (guanine/cytosine) over the entire 360 bp (Fig. 3) indicating a new variant genotype at the Irish sites. This sequence has a deletion at 20 bp from the 5′ end which is shared with the French strain (Arzul *et al*. 2001*a*, *b*) but was absent in the AY509253 and OsHV-1 μvar variants. In addition, the Irish OsHV-1 μvar variant genotype shows an insertion (guanine/cytosine) at 32 bp from the 5′end of the fragment, which is not present in any of the three French strains. In the Maximum Likelihood tree (Fig. 4), OsHV-1 μvar and the Irish OsHV-1 μvar variant genotype are closely related. The phylogenetic relationship between OsHV-1 μvar and the Irish OsHV-1 μvar variant genotype showed good bootstrap support (85%. *cf*. Fig. 4).

DISCUSSION

In this study, an undescribed Irish OsHV-1 μvar variant genotype was detected at 2 Irish *C. gigas* cultivation sites and was associated with on-going mortalities. The phylogenetic relationship among the 4 variants of OsHV-1 (Figs 3 and 4) shows that OsHV-1 μvar and the Irish variant genotype are most similar to each other. OsHV-1 μvar was described as a new OsHV-1 variant genotype based on 5 deletions (including a deletion of 4 microsatellite motifs; TAC) in the C region compared to OsHV-1. These deletions are also present in the Irish OsHV-1 μvar variant genotype with an additional 2 indels. These additional indels observed in the Irish variant genotype were not present in OsHV-1 μvar. Although, the 2 indels this study observed do not permit for strong conclusions about which virus variant is derived from which further sequence information is needed before a strong analysis of the relationship between OsHV-1 μvar and the Irish variant genotype can be established. However, the presence of 2 additional indels in the Irish OsHV-1 μvar could suggest that it may be derived from OsHV-1 μvar and represent a new variant of the virus. Alternatively, OsHV-1 μvar is derived from the Irish OsHV-1 μvar variant genotype, although that would demand that the 2 indels observed in the Irish variant genotype are ancestral for the clade containing OsHV-1 μvar and the Irish variant genotype, and have consequently been lost in OsHV-1 μvar. It is not clear if the Irish OsHV-1 μvar variant genotype differs from OsHV-1 μvar in virulence, tissue tropism etc. and it is obvious that more research is needed to confirm any biologically significant differences between the variant genotypes. Nevertheless, the close resemblance to OsHV-1 μvar in oysters sampled prior to 2008 in France (Segarra *et al*. 2010) may

indicate that OsHV-1 μvar has been present for longer than thought, but had escaped detection until 2008. While the Irish OsHV-1 μvar variant genotype represents a previously undocumented strain, there is no information of its biology being different than that of OsHV-1 μvar and future studies should examine additional areas of its genome and study the pathology to elucidate potential biological differences among the strains/genotypes. Herpes-like particles were detected by chance in a marine fungoid protist (thraustochytrid-like organism) (Arzul *et al*. 2001) and it has been suggested by Hurst *et al*. (2011) that marine protists may possibly act as vectors and/hosts for OsHV-1. A higher number of viruses are found in shallower coastal waters as more host organisms are available (Suttle, 2007).

In this study, an Irish OsHV-1 μvar variant genotype was detected in both adult *C. gigas* and *C. gigas* seed and juveniles. A lower percentage of seed and juveniles was observed to be infected compared to the adult oysters; however, the seed and juveniles had only been exposed for several weeks and months while the adult *C. gigas* had been at the Irish site for several years and screening indicated that no OsHV-1 or variant sequences were detected in initial samples of spat screened prior to relaying. It has been reported that *C. gigas* older than 18 months can be a source of the OsHV-1 and variants (European Food Safety Authority, 2010). Previous studies have shown that OsHV-1 can persist in surviving adult *C. gigas* following primary infection (Arzul *et al*. 2002; Lipart and Renault, 2002; Renault, 2008; Hurst *et al*. 2011) and it is recommended that movements and mixing of different age groups should be avoided (European Food Safety Authority, 2010).

The mortalities at both sites were associated with a complex aetiology including elevated temperature, nutrient and plankton levels (Malham *et al*. 2009; Cotter *et al*. 2010). Malham *et al*. (2009) demonstrated that oysters exposed to the environmental conditions recorded in this study were immunocompromised. A combination of these unfavourable environmental factors may have made the oysters more susceptible to the Irish OsHV-1 μvar variant genotype and eventually succumb to infection.

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