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# Ostreid herpesvirus OsHV-1 μVar in Pacific oysters *Crassostrea gigas* (Thunberg 1793) of the Wadden Sea, a UNESCO world heritage site

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## Abstract

The Wadden Sea is an extensive wetland area, recognized as UNESCO world heritage site of international importance. Since the mid-1990s, the invasive Pacific oyster *Crassostrea gigas* (Thunberg 1793) population in the area has grown exponentially, having a distinct impact on the ecosystem. The recent spread of the emerging oyster pathogen Ostreid herpesvirus OsHV-1 μVar worldwide and specifically in the oyster culture areas in the south of the Netherlands raised the question whether the virus may also be present in the Wadden Sea. In the summer of 2012 juvenile Pacific oysters were collected from five locations in the Dutch Wadden Sea. The virus was shown to be present in three of the five locations by real-time PCR and sequencing. It was concluded that OsHV-1 μVar has settled itself in Pacific oyster reefs in the Wadden Sea. These results and the recent discoveries of OsHV-1 microvariants in Australia and Korea indicate that OsHV-1 μVar and related variants might be more widespread than can be deduced from current literature. In particular in regions with no commercial oyster culture, similar to the Wadden Sea, the virus may go undetected as wild beds with mixed age classes hamper the detection of mortality among juvenile oysters.

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*Keywords: Crassostrea gigas*, molecular detection, mollusc health, oyster herpesvirus, viral disease, *Wadden Sea*.

## Introduction

The Wadden Sea is an extensive wetland area characterized by large intertidal flats, stretching from the Netherlands to Denmark. It is recognized as UNESCO world heritage site of international importance. The ecosystem in this area has an important role in a wide range of functions, varying from being a nursery area for commercially important fish and shrimp to providing a staging area for waterbirds migrating along the East-Atlantic flyway (Compton *et al.* 2013). The Pacific oyster *Crassostrea gigas* (Thunberg 1793) was first recorded in the Dutch Wadden Sea in 1983. Since then, the *C. gigas* population has grown exponentially from the mid-1990s onwards (Fey *et al.* 2009). The oysters are predominantly settling on intertidal blue mussel (*Mytilus edulis*) beds, which are increasingly transformed into *Crassostrea* reefs (Markert *et al.* 2013). The introduction of *Crassostrea gigas* has a distinct impact on the Wadden Sea ecosystem, among which a change in the foraging habitat of different bird species (Markert *et al.* 2013).

Mass mortalities of oysters are well-known phenomena occurring in different geographical regions for which various factors may play a role, including environmental factors (Petton *et al.* 2013; Green *et al.* 2014) and pathogens. Mass

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mortalities of adult Pacific oysters have occurred in the past in the Wadden Sea (Watermann *et al.* 2008; Fey *et al.* 2009; Büttger, Nehls & Witte 2011), usually linked to environmental causes like relatively warm summers or cold winters. The Pacific oyster is known to be susceptible to pathogens such as *Vibrio* spp. (Saulnier *et al.* 2010; Vezzulli *et al.* 2010) and Ostreid herpesvirus type 1 (OsHV-1; Nicolas, Comps & Cochennec 1992; Hine, Wesney & Hay 1992). Recently, a new genotype of oyster herpesvirus, OsHV-1 μVar, was detected and associated with high mortalities, locally over 95%, of juvenile *C. gigas* (Segarra *et al.* 2010). OsHV-1 μVar was first detected in France in 2008 and quickly spread over the main *C. gigas* culture areas in Europe. The virus was recorded along the coast of France (Segarra *et al.* 2010), Ireland (Peeler *et al.* 2012), England (OIE 2010), Spain (Roque *et al.* 2012), Italy (Dundon *et al.* 2011) and the Netherlands (Engelsma 2012) while outside Europe related OsHV-1 microvariants were detected in Australia (Jenkins *et al.* 2013) and Korea (Hwang *et al.* 2013). Infection with OsHV-1 microvariants has been recently listed as a notifiable disease for the World Organization of Animal Health (OIE 2014). In the Netherlands OsHV-1 μVar has been detected since 2010 in Lake Grevelingen and the Oosterschelde, two Pacific oyster culture areas in the south-west of the Netherlands (Engelsma 2012). In the Dutch Wadden Sea, mortalities of juvenile Pacific oysters that could be linked to the presence of OsHV-1 μVar have not been reported. However, mortality of juvenile oysters is difficult to assess in wild beds of mixed age classes. In 2011, a preliminary attempt has been carried out to investigate the presence of OsHV-1 μVar in the Wadden Sea with a very limited number of samples from Texel and Sylt (Thieltges *et al.* 2013). OsHV-1 μVar could not be demonstrated in the samples at that time.

Taking into account the recent spread of the virus and its presence in the oyster culture areas in the Netherlands, it was questioned whether OsHV-1 μVar could possibly be present in the oyster reefs in the Wadden Sea and if so, what the effect would be on the Pacific oyster population. To address the first question five oyster reefs in the Dutch Wadden Sea were screened in 2012 for the presence of OsHV-1 by real-time PCR. The OsHV-1 genotype was subsequently determined by sequencing of two OsHV-1 genome regions from positive samples.

## Materials and methods

Samples were taken from five locations (see Fig. 1), selected to represent the various habitats in which oyster reefs are found in the Wadden Sea. They differed in being either sheltered or exposed, and in being either a reef dominated by Pacific oysters, *Crassostrea gigas*, or being a more mixed shellfish bed, which also included blue mussels, *Mytilus edulis* (L.) and common cockles, *Cerastoderma edule* (L.) (Table 1).

As outbreaks of OsHV-1 μVar are reported at temperatures above 16 °C (Dégremont *et al.* 2013), the optimal temperature for the virus appears to be above this temperature. Hence, the sampling was conducted at the end of the summer in the last week

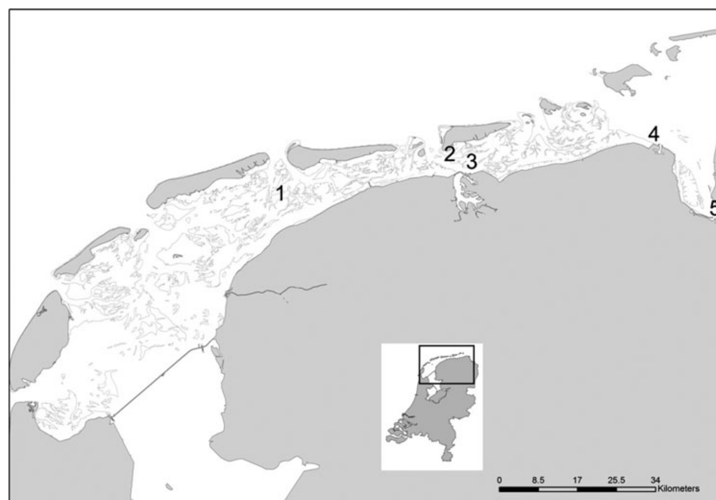


**Figure 1** Locations sampled: 1, Het Abt; 2, Schiermonnikoog; 3, Vierhuistergat; 4, Eemshaven; 5, Termunterzijl.

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**Table 1** Sampling locations, habitat descriptions of the locations, number of Pacific oysters tested from each location and results of real-time PCR analysis on OsHV-1

| Location | | Location description | # Pacific oysters tested | # Tested positive for OsHV-1 |
|---|---|---|---|---|
| 1 | Het Abt | Mixed shellfish bed with mainly oysters, cockles and mussels | 30 | 0 |
| 2 | Schiermonnikoog | Large shellfish bed with small tidal pools that lie close to the island. locally dominated by either mussels or oysters | 30 | 9 |
| 3 | Vierhuistergat | Seaweeds, oysters and mussels lying scattered around a jetty. Oysters of all ages were present, but the larger ones were rare | 30 | 3 |
| 4 | Eemshaven | The sheltered side of a riprap dike in the harbour, mostly overgrown by oysters of all sizes. A riprap dike is a loose assemblage of broken stones erected in water as a foundation | 30 | 8 |
| 5 | Termunterzijl | A jetty, locally covered with seaweeds. Large numbers of oysters of which only a few were found to be alive | 17 | 0 |

of August and the first weeks of September of 2012, when water temperatures in the Wadden Sea are at their annual highest (around 20 °C).

At each location, 30 juvenile oysters (<2.0 cm shell length) were collected, with the exception of the location Termunterzijl where only 17 individuals were collected, as most of the oysters at that location appeared to have died recently by unknown causes. At the other locations, no abnormal mortalities were observed. All 137 individuals were screened for the presence of OsHV-1 μVar by real-time PCR. In brief, after collection, the samples were frozen and stored at −20 °C until further processing. Of each individual oyster, the soft tissue was homogenized with a TeSeE PRECESS 24 homogenizer (Bio-Rad) in tissue disruption tubes equipped with ceramic beads. Total DNA was extracted using the automated MagNa Pure LC system (Roche). The TaqMan real-time PCR was performed using the primers and probes as described by Martenot *et al.* (2010): OsHV1 BF (5′-GTCGCATCTTTGGATTTAACAA-3′), reverse B4 (5′-ACTGGGATCCGACTGACAAC -3′) and the probe B (5′-TGCCCCTGTCATCTT GAGGTATAGACAATC-3′). The real-time PCR consisted of 1× TaqMan Fast Universal PCR Master Mix (Applied Biosystems) including 0.4 μM of each primer, 0.2 μM probe, 1.25 units of Uracil-DNA Glycosylase (New England BioLabs) and 5 μL of template DNA to a final volume of 20 μL per reaction. The real-time PCR was carried out in an AB 7500 with fast block (Applied Biosystems) according to the program: 37 °C for 10 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Prior to testing of the samples, the limit of detection (LOD) of the assay was assessed by a 1/4 serial dilution of an OsHV-1 μVar-positive field sample tested in eightfold. Based on this, the LOD of the assay was estimated to be around cycle threshold (Ct) 36.5. This value was subsequently used as cut-off value for the assay.

The genotype of OsHV-1 was determined by amplification and direct sequencing of the C2/C6 region and the IA1/IA2 region from positive samples according to Segarra *et al.* (2010). In brief, the conventional PCR mixture consisted of 0.2 μM of each primer C2 (5′-CTC TTT ACC ATG AAG ATA CCC ACC-3′)–C6 (5′- GTG CAC GGC TTA CCA TTT TT-3′) or IA1 (5′-CGC GGT TCA TAT CCA AAG TT-3′)– IA2 (5′-AAT CCC CAT GTT TCT TG CTG-3′) (Eurogentec), PCR buffer at 1× concentration, 2.5 mM $MgCl_2$, 200 μM of each deoxynucleotide (dNTP mixture, Takara Bio Inc.), 2 units Taq DNA Polymerase (Invitrogen) and 5 μL of template DNA. The thermal profile of the PCR was an initial denaturation at 94 °C for 2 min followed by 35 cycles at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 5 min. After purification with QIAquick PCR Purification Kit (Qiagen), the PCR products were sequenced in both directions using an ABI Prism BigDye v 1.1 Terminator Cycle Sequencing kit (Applied Biosystems) and the corresponding amplification primers and analysed on an ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems).

## Results

As result of the analyses, juvenile Pacific oysters were found positive for OsHV-1 by real-time PCR at three of the five locations (Table 1). At the OsHV-1 positive sites, the number of oysters

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in which genomic material of OsHV-1 could be detected ranged from 3 of 30 to 9 of 30. For most positive samples, high Ct values were obtained, above Ct 30, indicative for a low amount of viral copies. This hampered subsequent genetic analysis. From only five samples (one from location 3 and four from location 2), sequences could be obtained by amplification and direct sequencing of the C2/C6 and IA1/IA2 region. The sequences from both genomic regions were 100% identical to sequences of OsHV-1 μVar as described by Segarra *et al.* (2010) and hence contained the characteristic substitutions and microsatellite zone of OsHV-1 μVar in both regions.

## Discussion

From these results, it can be concluded that OsHV-1 μVar has reached the Wadden Sea and OsHV-1 has settled itself in at least three Pacific oyster reefs. As the prevalence in each of these populations was low, it remains uncertain whether the virus was truly absent in the remaining two populations or was missed there by undersampling. Many recently dead oysters were recorded during the fieldwork at Termunterzijl where only 17 alive juvenile oysters could be collected. None of these individuals was tested positive for OsHV-1 μVar. However, due to the limited sample size, this does not necessarily exclude the presence of the virus as cause of the mortalities. The results indicate that the virus may be widespread in at least the Dutch part of the Wadden Sea.

In remains uncertain when the virus was introduced into the Wadden Sea. Considering the observation at distinct sites, the virus may have been present undetected for several years. In natural populations with Pacific oysters forming *Crassostrea* reefs, mortalities among spat and juveniles only will be difficult to detect. Empty shells of spat and juveniles are easily lost or overgrown by other organisms. For example, in 2011, OsHV-1 μVar was detected in Pacific oyster spat collected on disc collectors from two locations in Lake Grevelingen, the Netherlands (Engelsma 2012). Although no mortality was observed in wild beds of Pacific oysters at the same locations, on the collectors,the cumulative mortality of juveniles ranged up to 46%.

The route of introduction of the virus into the Wadden Sea remains unknown. Transport of the infected host is one of the major risk factors in spread of disease (Murray *et al.* 2012). However, the only recent oyster imports from abroad (Britain) into the Wadden Sea took place in the German part, north of Sylt (Moehler *et al.* 2011). This area is a few hundred kilometres up-current from the Pacific oyster populations that were found to be infected and there are no indications that oysters have recently been transported from north to south in the Wadden Sea. Other possible routes of introduction into the Wadden Sea are via infected oysters attached to ship hulls or transfer with planktonic larvae of the Pacific oyster along the general south–north current of Dutch coast. With regard to the latter, this might be possible either directly, as larvae can drift along in the sea-currents for 2–4 weeks (Rohfritsch *et al.* 2013), or indirectly via settled oysters on hard substrates like embankments, buoys and structures in harbours and off-shore windmill parks that function as stepping stones in between the Delta and the Wadden Sea.

The impact of OsHV-1 μVar on the *C. gigas* population in the Wadden Sea could not be deduced from this study. Possibly, losses by OsHV-1 μVar can be compensated by the massive spat fall of *C. gigas* in the area, as observed over the last decade, but this would be a subject for further studies. Thus far, OsHV-1 μVar has been primarily detected in the culture areas in Europe. In wild populations, observations of mortalities among spat and juvenile molluscs present difficulties. The present results indicate that OsHV-1 μVar might be more widespread than can be concluded from records in literature alone. Similarly to the Wadden Sea, the virus may go undetected especially in regions with no commercial oyster culture.

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