Ministry for Primary Industries
Manatū Ahu Matua

Ostreid herpesvirus and *Vibrio* species pilot surveillance study farmed Pacific Oysters (*Magallana gigas*) in Croisilles Harbour, Marlborough Sounds, New Zealand.

MPI Technical Paper No: 2019/01

By Anjali Pande (Principal Adviser/ Incursion Investigator, SIIAEH),
Mary Van Andel (Principal Adviser/ Veterinary epidemiologist, SIIAH)

ISBN No: 978-1-98-857173-7 (online)
ISSN No: 2253-3923 (online)

21 December 2018

New Zealand Government

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Requests for further copies should be directed to:

Publications Logistics Officer
Ministry for Primary Industries
PO Box 2526
WELLINGTON 6140

Email: brand@mpi.govt.nz
Telephone: 0800 00 83 33
Facsimile: 04-894 0300

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Executive Summary

A pilot surveillance programme was run between 2016 - 2018 with collaboration between oyster farmers and the Ministry for Primary Industries (MPI) to examine feasibility of running such a programme, and what would make it possible and valuable.

We identified that timing, design and frequency of sampling events are very important and that communication between the laboratory and the field staff is crucial for diagnostics to operate smoothly. This pilot programme with the chosen pathogens: Ostreid herpesvirus-1 microvariants (OsHV-1) and *Vibrio* sp. was not able to act as an early warning system for potential mortality events based on the sampling frequency trialled in this study.

The prevalence of OsHV-1 and the five target *Vibrio* sp. (*V. aestuarianus*, *V.harveyii*, *V. parahaemolyticus, V. splendidus* and *V. vulnificus*.) was found to be low in the study site.

Initial data analysis suggests that rainfall and phytoplankton biotoxins may be important risk factors in determining increased OsHV-1 prevalence, but further work is needed to properly understand this relationship.

Introduction

New Zealand currently has no structured aquatic animal health surveillance system. To develop a system for all of New Zealand, especially in the aquaculture area, will be a long term step by step process. Currently the lack of an industry-wide aquatic animal health surveillance system, beyond the MPI Pest and Disease Hotline (0800 80 9966), increases the risk of a new pathogen going undetected for longer than with an active surveillance programme and therefore reduce the ability to respond to it effectively.

In order for any surveillance system to function effectively, buy-in is needed from the industry sector, even if the system is regulated/legislated. A comprehensive surveillance system requires resourcing. Ideally, industry and government need to work together to build a system that benefits all parties.

In this project MPI worked alongside a specific industry sector, Pacific Oyster farmers, in a small scale pilot study. The purpose was to test the feasibility and value of regular surveillance and demonstrate how it could work as a means of an early warning system for emerging health issues.

PACIFIC OYSTER FARMING INDUSTRY

The pacific oyster is one of the three main aquaculture species in New Zealand and has a large export market. In 2009, New Zealand's pacific oyster industry was significantly affected by the presence of the Ostreid herpesvirus-1 microvariants (OsHV-1).

As a result of this impact, some pacific oyster farmers have modified their farming practices to try and mitigate the damage or effects caused by OsHV-1. However, they are still trying to understand the distribution and extent of OsHV-1 and to establish the risk factors for clinical disease. To this end, a group of oyster farmers agreed to work with MPI on a pilot study to test a health surveillance system.

OBJECTIVE

To establish a baseline health status in pacific oyster in Croisilles Harbour and set up a trial surveillance programme as an early warning system for emerging disease.

For Oyster Farmers

1) A way to help protect the oyster farmers and avoid or reduce impacts such as those seen in the OsHV-1 outbreak in 2010/11
   a) Develop a "health certificate". This should be available at the point of the oysters leaving the sea farm in the South Island as a business advantage for oyster industry partners

2) Determine the presence/ absence of OsHV-1 in Croisilles Harbour

For MPI:

1) Test feasibility of a sampling/ surveillance protocol
2) Identify gaps in how this type of surveillance system might work
3) Gain information that may benefit Government Industry Agreement (GIA) discussions

For All:

As part of the data gathered from this pilot study we hoped to examine correlations of OsHV-1, environmental parameters and *Vibrio* species, to add to the knowledge about how OsHV-1 manifests as a clinical disease.

Methodology

GEOGRAPHICAL AREA

The study was limited to Pacific oyster marine farms within Croisilles Harbour, in the outer Marlborough Sounds on the west coast of New Zealand's South island.

Fig.1. Location of Croisilles Harbour in Marlborough Sounds.

COSTS

The costs for this project were shared between MPI and the oyster farming Industry. The farmers collected and provided the oysters on a pre-agreed schedule and organised shipping to the chosen diagnostic laboratory as well as providing environmental data for the duration of the project. MPI arranged diagnostic testing, analysed the results, did the reporting and managed the project.

TIMING

The project commenced in October 2016 and was to run for two years until October 2018.

PATHOGENS

The agreed pathogens to test for were;

- Ostreid herpesvirus 1 microvariants (OsHV-1)
- *Vibrio* spp. (those known to be of significance to aquatic animal health, or could affect the industry specifically due to there being associated human health issues. The five target vibrio species were; *V. aestuarianus*, *V.harveyii*, *V. parahaemolyticus, V. splendidus* and *V. vulnificus*.).

GENERAL SAMPLING STRATEGY

Sample collection

- Sampling was designed to maximise detection of OsHV-1, therefore the sampling was weighted towards summer. Four sampling events per year (3 in summer, 1 in winter).

- Samples were taken around grading time + ~3 days - Preferentially samples collected should be of;
    - Runts
    - Small, old animals or
    - Young animals
- Sample size per sampling event was 150 oysters.

- Oyster samples were collected on pre-organised dates from one particular farm. Given oysters are moved between farms within the bay, on a regular basis during the grading process, it was thought that a sample taken from any given farm would be representative of the whole bay. Where there were runt oysters (oysters that had not grown to commercial size, sometimes left over from the previous season) these were collected as part of the sample. Ideally 75 runt oysters and 75 juvenile oysters were collected at each sampling event. In 2018, there were no runt oysters available so 150 juveniles were collected.

- Environmental parameter data collected/ collated
    - Water temperature (collected from a data logger on the farm)
    - Rainfall (from existing rain gauge information)
    - Phytoplankton levels (from Marine Shellfish Quality Programme (MSQP) - http://www.marinefarming.co.nz/quality.asp

Diagnostics

The samples were sent to an MPI approved contracted laboratory for diagnostic testing. The oysters were all tested for the presence of OsHV-1 (using Real time Polymerase Chain Reaction (PCR) molecular tests, Martenot et al. 2010). The cycle threshold (Ct) value was used as an estimate of the quantitative amount of OsHV-1 particles or viral copy numbers present in the tissue. Ct levels are inversely proportional to the amount of target nucleic acid in the sample (ie: the higher the Ct value, the more indicative of a low amount of virus present.)

Presence of the target *Vibrio* spp was also recorded (identified by MALDI-TOF). Only 20 plates from each sampling event had bacteriology performed on them as the budget did not allow for diagnostic analysis of all bacterial plates, but where possible, plates from any oysters that were OsHV-1 positive would be re-examined for presence of *Vibrio* spp.

The 20 plates selected had a representative (by visual inspection) collection of bacterial colonies on them, and these were subjected to MALDI-TOF for species identification. If the target species were not identified, any other dominant colonies were identified and documented. Detailed testing methods are described in the report to MPI (Wong and Engelander, 2018).

Data on phytoplankton and biotoxins were provided by the Marlborough shellfish quality programme (MSQP), as was rainfall and harvesting closures data. Water temperature data were obtained from a temperature logger attached to the marine farm itself, which meant these data were accurate at a small spatial scale.

The top five species of phytoplankton for producing biotoxins that were considered in this analysis were; *Pseudonitzschia spp., Dinophysis acuta or accuminata, Karenia mikimotoi, Gymnodinium spp.. and Alexandrium, spp*.

DATA ANALYSIS

When analysing the presence of OsHV-1 the key outcome variable used for analysis was the Ct value (which is a quantitative measure of the amount of virus present in the tissue). If the quantitative value itself was not used in the analyses, the Ct value was used to simply indicate presence of OsHV-1. The explanatory variables were the environmental factors for which we collected information. These were water temperature, rainfall, phytoplankton/ biotoxins and presence and level of *Vibrio* spp. and other bacteria. Where appropriate statistical t-tests were also performed.

An exploratory data analysis was performed using these variables. This included box plots; two by two tables and a general graph (Fig. 1) to examine associations. After this exploratory data analysis was complete, logistic regression models were built and odds ratios calculated to assess statistical significance of association.

Any significant factors were included in a multivariable model.

Results and Discussion – Operational Evaluation

FEASIBILITY

The sampling protocol was able to be carried out by the oyster farmers and samples sent to the diagnostic laboratory on time for the most part. On occasion the lab was ready to receive samples but they did not arrive until later, impeding laboratory workflow. The lesson learned from this is that pre-arranged sampling dates and communication of any delays is very important, as it impacts the laboratory proceedings to process samples.

During development of the sampling protocol no field visits were undertaken until later in the project. This meant that communications about any substitutions and changes to sampling that may have been necessary due to logistics, were hard to understand and interpret by non-field staff. This highlights the importance of undertaking a field visit as part of the sampling protocol development.

The diagnostic results were generally delivered within the 10 working days. However this often needed adjustment or re-evaluation, so the final results, with interpretation from MPI staff, were often not delivered to the farmers until later. If this programme had been set up with a sampling frequency adequate as an early warning system, this would have lost its efficacy when results were delayed. This highlights the importance of regular communication and potentially having a system for interim verbal results before the final results are released.

The training of another lab outside of the MPI National Animal Health Laboratory (AHL) was successful and therefore MPI have developed capability for testing of an aquatic animal disease in New Zealand. Further developing this capability throughout New Zealand is very important as it lightens the load on AHL and means that farmers have other diagnostic avenues available to them. One aspect that would need to be considered is the relationship between a diagnostic laboratory and farmers without the benefit of an experienced aquatic animal health professional helping to interpret the results and offering advice around these results.

GAPS

The pilot study, in its current form, was unable to act as an early warning system for this pathogen. However, this project demonstrated that a targeted surveillance programme could work well, in the sense that having pre-organised sampling allows for successful collection and testing of oysters in a timely fashion.

EARLY WARNING

A functional early warning system requires more frequent sampling events. These should be timed based on existing husbandry practices and knowledge of the pathogen and its interaction with environmental conditions. There was a large mortality event between sampling periods (sampling occurred in early December and early February, and the mortality event occurred around December 24, 2017). The early December sampling period did not show any OsHV-1 present in the sample, but the February sample showed the highest prevalence during this programme. This could be expected based on what is known about the behaviour of OsHV-1 in susceptible populations. However, this indicated that the testing undertaken was not useful as a predictor for this mortality event. To be of benefit to oyster farmers, this would be a key point. To be useful as a predictor, it is likely to need an increased frequency of sampling, especially around peak risk periods. This could be in the form of something like a trigger point, such as a well-defined rapid change in temperature, or reaching of a certain water temperature instigating additional sampling.

It might be useful for a targeted surveillance plan to have a contingency in place to examine mortality events outside of the regular sampling protocol. This may be more responsive and provide faster turnaround of results than utilising the MPI 0800 exotic pests and diseases hotline. In this case the MPI passive surveillance system (exotic pest and disease hotline) was not contacted, as the lab was closed over the Christmas period and therefore no samples were able to be tested. This testing time delay, would not be useful for informing any action by the farmers. Samples adjacent to the pilot study sampling site were showing signs of mortality during the January sampling event. These were examined separately and there were no positive results for OsHV-1.

However, these difficulties may be specific to the pathogen tested (i.e. OsHV-1). Ideally a general health screen (histology) should be considered, especially for providing the oyster farmers with a “health certificate”. This was not done for this pilot programme due to potential trade issues should certain pathogens be identified. To generate a health certificate that is benefit to the wider industry, the testing programme needs to take into account the complexity of the environment and develop the sampling strategy in accordance with this. Additionally, a health certificate is really only useful if there is intent to move stock between areas with a different health status, or between areas where there might be other susceptible species. The health certificate can then be used to demonstrate biosecurity best practice has been followed in the case of a pathogen being found in a new area, or in a new host species. However, it is unlikely to be of interest to a consumer, and therefore would not benefit industry as a marketing tool. It may be worth considering that in the future a health certificate may be useful for export requirements of international markets.

The testing carried out in this pilot study, with a higher sampling frequency and therefore a higher level of certainty, would be useful prior to the transfer of stock from area to area, to prevent moving pathogens around the country. This is recommended aquaculture best biosecurity practice, but not a legal requirement.

PRESENCE/ ABSENCE OF OSHV-1 IN CROISILLES

This trial surveillance programme did indicate that OsHV-1 is present in the Croisilles Harbour at a very low prevalence (see data analysis sections).

It was suggested that despite moving oysters around, and the sampling protocol theoretically being indicative of the whole bay, the sampling protocol should be designed differently to develop a better picture of the spatial movement of virus throughout the bay. This could be an area of future research involving hydrodynamics and looking at small scale differences in water movements to help inform the pattern of spread of the virus over varying spatial scales. This in turn would help inform growing management of the oyster industry. Alternatively developing a protocol with set trigger levels (e.g. certain water temperatures) to initiate testing, could potentially be applied nationally.

Results – Data Analysis

GENERAL OVERVIEW

A general overview appears to show a relationship with high water temperatures and presence of OsHV-1. There are no other clear patterns. At the start of the sampling programme it was only the runt oysters that showed presence of OsHV-1, later on when only juveniles were collected, some of them were positive for OsHV-1. A high prevalence of the virus (57.3%) was seen in February 2018, at the same time as there was a big spike in water temperature.

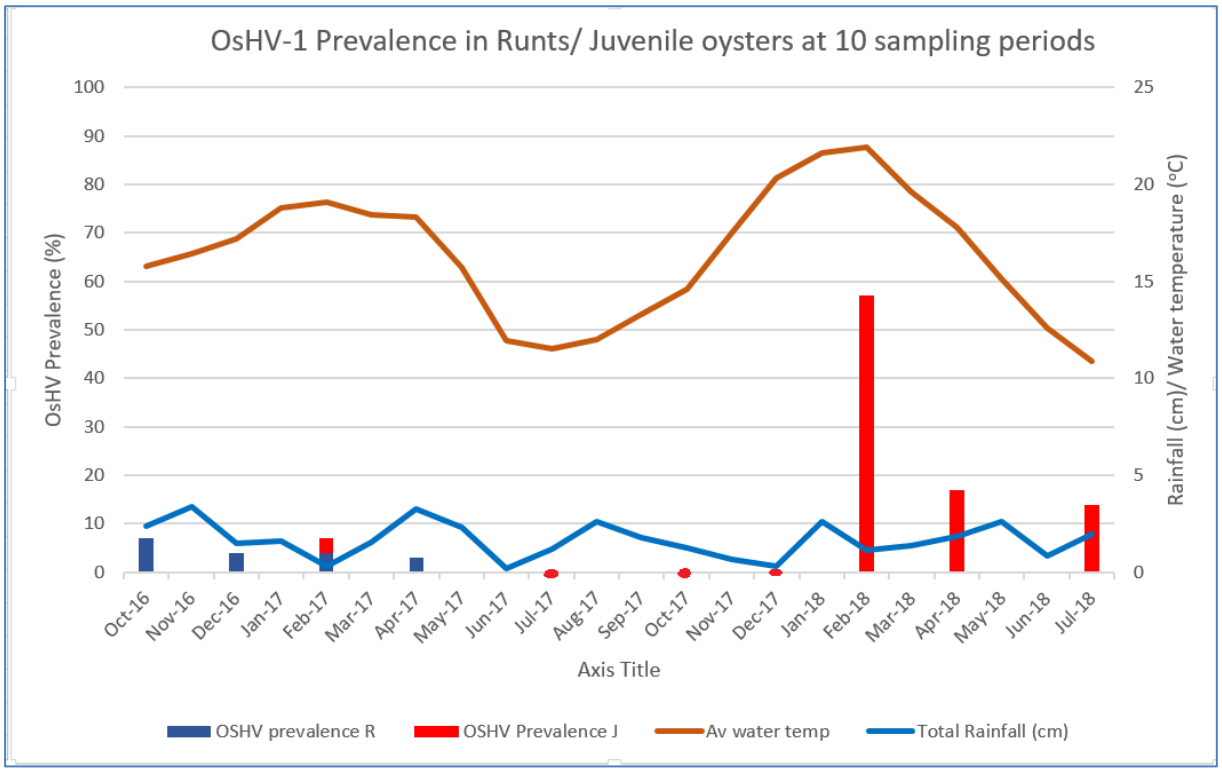

Fig 2. OsHV-1 prevalence in runts and juvenile oysters over the 10 sampling periods, with water temperature and rainfall.

PREVALENCE OF OSHV-1

Over 10 sampling periods, the highest prevalence of OsHV-1 seen was 57.3% of a sample size of 143 oysters (82 oysters) as is seen in Table 1.

Table 1. OsHV prevalence over the 10 sampling periods.

Prevalence Table				
year	Month	Runts tested (# positive)	Juveniles tested (# positive)	Total Oysters tested (# positive/ prevalence %)
2016	Oct	75 (5)	75(0)	150 (5/ 3.3 %)
2016	Dec	74 (3)	76 (0)	150 (3/2 %)
2017	Feb	75 (3)	75 (2)	150 (5/ 3.3 %)
2017	April	75(2)	75(0)	150 (2 / 1.3 %)
2017	July	75(0)	75(0)	150 (0/ 0%)
2017	Oct	0	150(0)	150 (0/ 0%)
2017	Dec	0	100 (0)	100 (0/ 0%)
2018	Feb	0	143 (82)	143 (82/ 57.3%)
2018	April	0	150 (26)	150 (26 /17.3%)
2018	July	0	150 (21)	150 (21/ 14 %)

The lowest recorded Ct value was 27.2, the highest recorded Ct value was 39.23, and the mean value (excluding 0 values) of 33.93. Zero values were excluded because they would artificially lower the average Ct value where the virus was actually present.

Ct < 29 are strong positive reactions indicative of abundant target nucleic acid in the sample
Ct = 30-37 are positive reactions indicative of moderate amounts of target nucleic acid
Ct = 38-40 are weak reactions indicative of minimal amounts of target nucleic acid which could represent an infection state or environmental contamination.

In the early half of the surveillance programme both runts and juveniles were collected and the OsHV-1 prevalence was, on average, lower in the juveniles as seen in table 1. See box plot (Fig. 3), which shows the Ct values were lower in juvenile oysters, indicating a higher quantity of virus.

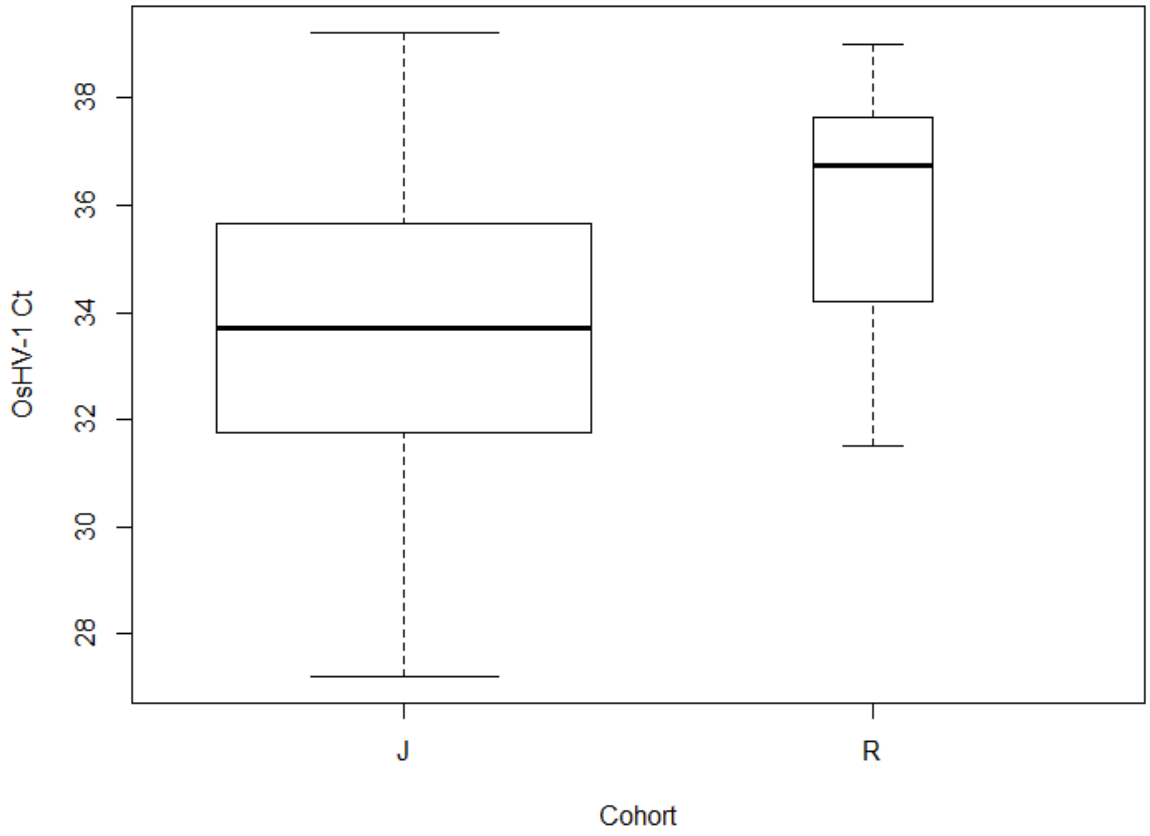

Fig 3. Difference in OsHV-1 Ct values in runt and juvenile oysters. The average Ct values in the juvenile oysters are lower, indicating a higher quantity of virus. Box size indicates the relative sample size of each cohort over the course of the study. After 2017, only juvenile oysters were available to be sampled.

VIBRIO OR BACTERIA AS RISK FACTORS

Target *Vibrio* spp. that had been tested for were not common. *Vibrio parahaemolyticus* was only found once and *Vibrio harveyii* was found three times. Out of those oysters positive for OsHV-1, only one oyster tested positive for one of the five target *Vibrio* spp, which was *Vibrio harveyii*.

Three analyses were carried out: each Target *Vibrio* spp was examined separately in relation to OsHV-1 presence; all target *Vibrio* spp were also grouped and tested in a similar manner; all *Vibrio* bacteria identified were also grouped (both including and not including the target *Vibrio* spp. in the grouping) and tested against OsHV-1 presence. None of these groupings showed any significant (or close to significant) difference in prevalence of OsHV-1.

Performing t-tests showed no significant difference (target *Vibrio* spp present (p = 0.654)/ all bacteria grouped (p = 0.615)/ All other bacteria excluding target *Vibrio* spp. (p = 0.615). This could be attributed to sample size.

RAINFALL AS A RISK FACTOR

The relationship between OsHV-1 prevalence and rainfall was examined in two ways. The total rainfall in the month prior to sampling was examined. Freshwater flushing specifically was measured in two ways. If severe rainfall and freshwater flushing closed the bay to shellfish harvesting in the month preceding sampling (as per MSQP requirements) this was noted, and if this was the case, the number of days it had been closed was also counted. All sampling periods had some high water flushing and closures in the month prior, so this was excluded from the analysis.

Fig. 4 shows that more days of harvest closures due to rain, resulted in a higher average count of OsHV-1 positive results. Higher total rainfall in the preceding month showed a similar pattern. Table 2 shows that the odds of detecting OsHV-1 was increased by 1.004 times for each mm unit of increase in rainfall, or the odds of detecting OsHV-1 was increased by 1.175 times for each additional day of closure due to rainfall.
These two measures of rainfall were not included in the same model as they are basically different ways of describing the same thing, with the subtle difference of closures being indicative that the total amount of rainfall happened over a short period of time rather than being spread evenly throughout the month.

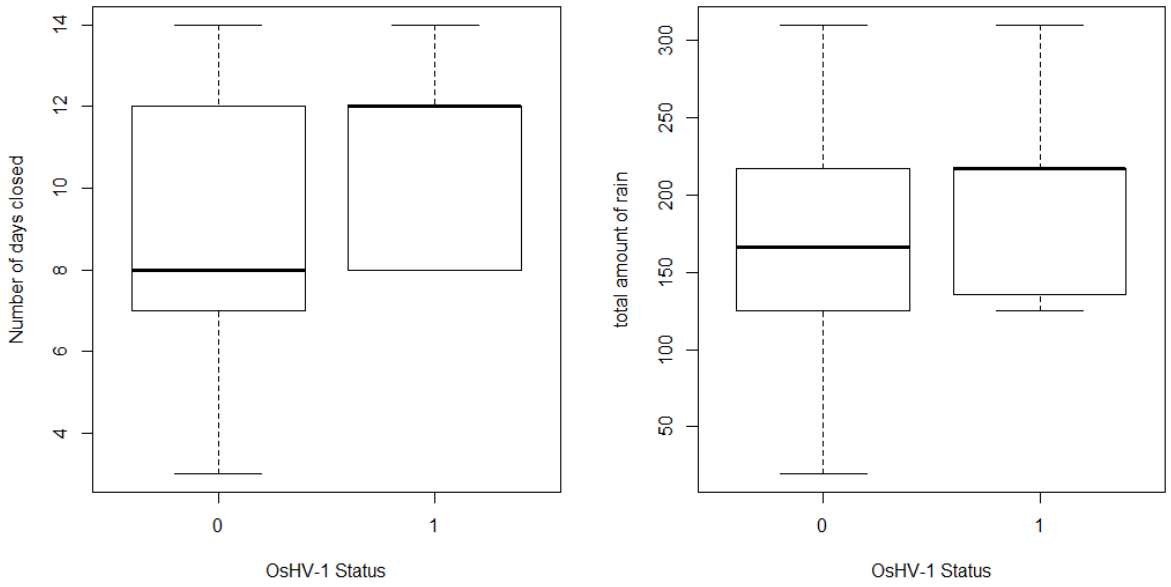

Fig. 4. Boxplot showing that the more days an area was closed due to high rainfall, or more total rainfall (mm) was more likely to trend toward a positive OsHV-1 result.

PHYTOPLANKTON/ BIOTOXIN AS A RISK FACTOR

Another factor looked at was phytoplankton and associated biotoxins. This was examined in two ways. Whether or not the top five important phytoplankton species were present in the preceding month and also whether or not this phytoplankton was present at a level that would trigger flesh testing in shellfish. All sampling periods had at least one of the top five species present in the preceding month, so this was excluded from the analysis.

OsHV-1 Ct values were plotted against whether or not any of the top five phytoplankton species were present in levels triggering flesh testing. The OsHV-1 Ct values were generally lower (indicating a higher level of virus) when phytoplankton were present in trigger levels.

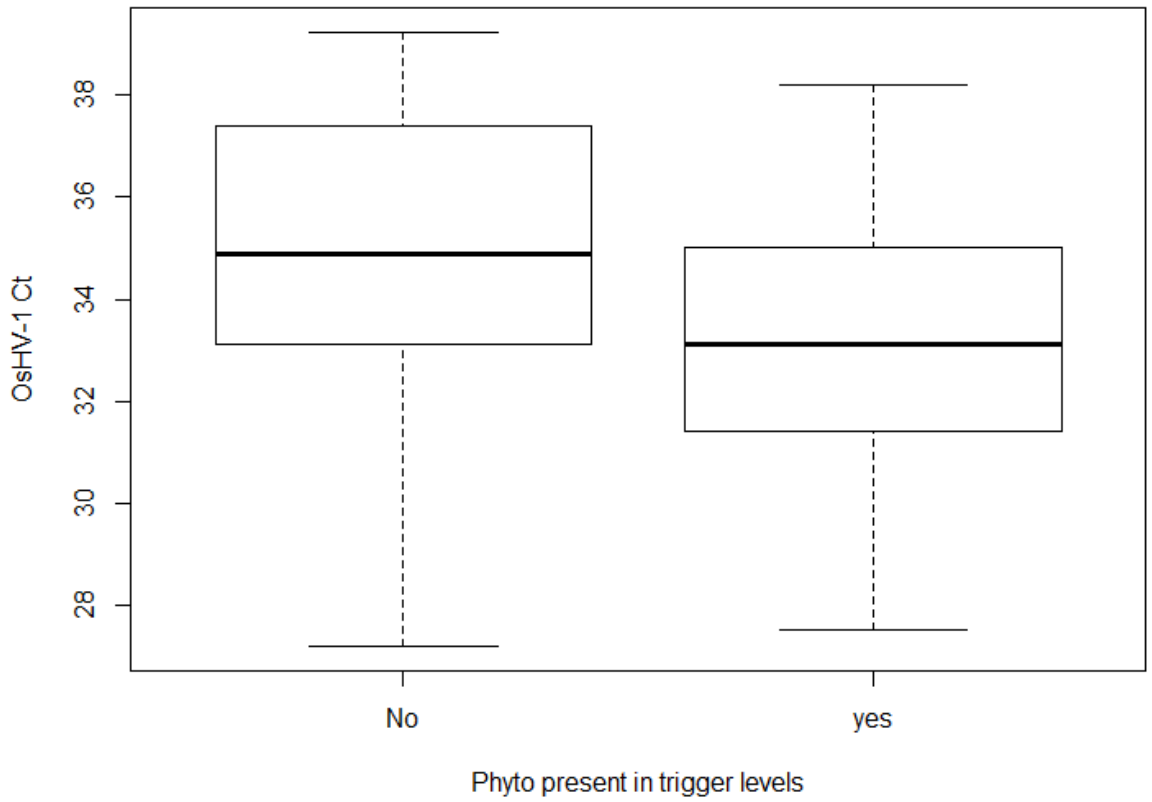

Fig.5. Boxplot showing that when any of the top five important species of biotoxin producing phytoplankton (*Pseudonitzschia spp., Dinophysis acuta or accuminata, Karenia mikimotoi, Gymnodinium spp.. and Alexandrium, spp*.) are present in levels that trigger flesh testing, the OsHV-1 Ct values are lower (indicating a higher quantity of virus)

A t-test also showed this difference to be significant (p = 0.000)

WATER TEMPERATURE AS A FACTOR

Water temperature was a significant factor increasing prevalence of OsHV-1. The boxplot (Fig. 6) shows that the boxes are not overlapping suggesting a significant difference). A t-test also showed a significant difference in the mean water temperature associated with a positive OsHV-1 result (p = 0.000).

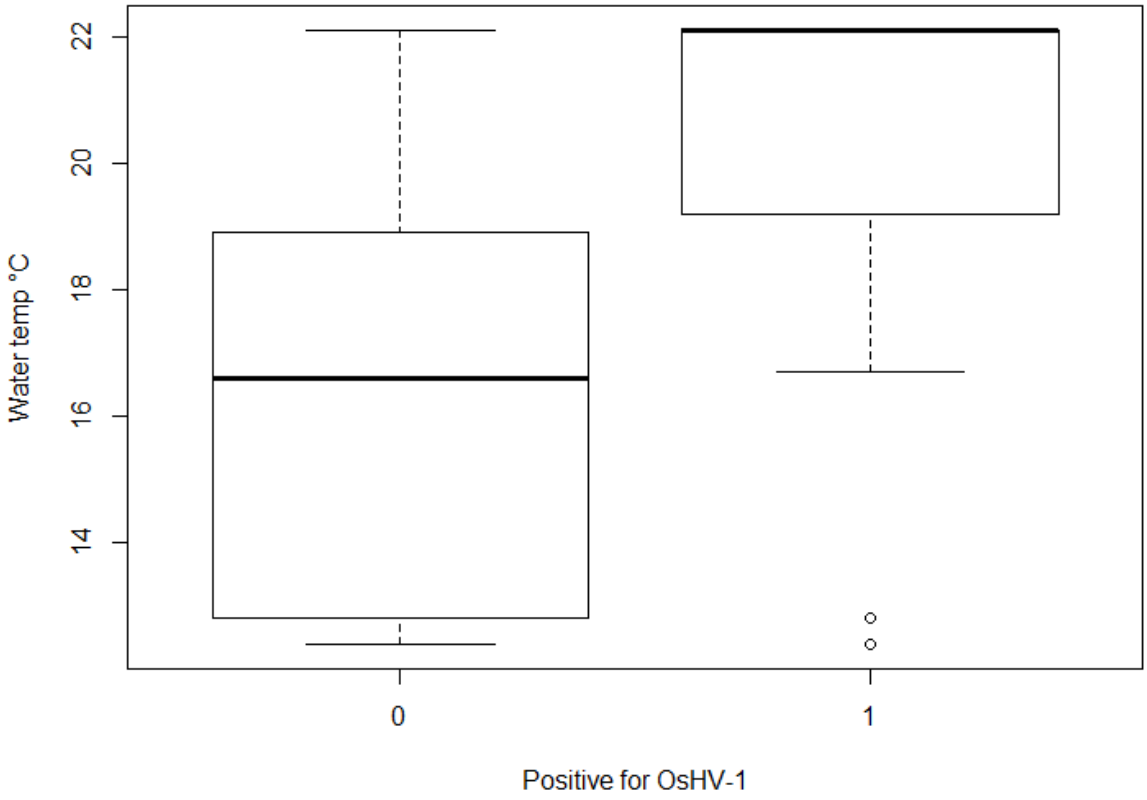

Fig. 6. Positive results for OsHV-1 presence are associated with higher water temperatures.

GENERAL LINEAR MODELS

General linear models show the same results.

Univariable logistic regression was performed for the factors that might be significant in explaining increased prevalence in OsHV-1 (Table 2).

The factors that were significant to be retained in the multivariable model were water temperature, rainfall and presence of any of the top five phytoplankton species in trigger levels.

Table 2. Results from the univariable models, showing the *p*-values, Odds ratios and confidence intervals for all the examined factors. *p*-values smaller than 0.2 were used in the multivariable model.

Factor	*p*-Value	Odds ratio	Confidence Interval
All Target *Vibrio* spp. grouped	0.516	2.13	0.104 -17.117
All bacteria grouped	0.796	1.253	0.337-8.139
Water temperature	0.000*	1.518	1.413 – 1.640
Rainfall – number of days closed	0.000*	1.175	1.111-1.245
Rainfall – total amount in preceding month	0.001*	1.004	1.002 – 1.006
Any of top 5 phytoplankton species present in trigger levels	0.000*	4.202	2.955 – 6.002

*= Significant statistical result

Testing for confounding and interaction

Testing the models for interaction and confounding indicated that water temperature is a confounding factor (i.e. can have an effect on the outcome variable and the other dependant variables). This means that water temperature must remain in the model. In the model where rainfall flushing was measured by days closed, presence of top five phytoplankton was also a confounder meaning it also needs to remain in the model.

No interaction terms were significant.

Table 3. Results from all the multivariable models tested. Total rainfall (mm) and days closed due to flushing were effectively two ways of measuring the same factor, therefore these were not put in the same model but two different models were built using one of these two measures of rainfall. A stepwise progression was used to build these models, dropping whichever factor was not significant in the total model. Water temperature was going to have to stay in the final model since previous tests showed it was a confounder, and when using number of days closed due to rainwater flushing in the model, presence of the top five phytoplankton in trigger levels was also significant. This was measured by the percent change in coefficients. The models marked with an (*) were only built for the purposes of checking confounding.

Model	Factor	*p*-Value	Retained	Change in Coefficient (%) from whole model
Model 1: Total Rainfall + Top 5 Phytoplankton + Water temperature	Total rainfall	0.105	No	NA
Model 1: Total Rainfall + Top 5 Phytoplankton + Water temperature	Top 5 Phytoplankton	0.000	Yes	NA

Model 1: Total Rainfall + Top 5 Phytoplankton + Water temperature	Water temperature	0.000	Yes	NA
Model 1.1 Top 5 phytoplankton + water temperature	Top 5 Phytoplankton	0.000	Yes	3.5
Model 1.1 Top 5 phytoplankton + Rainfall	Water temperature	0.000	Yes	4.9
*Model 1.2 Top 5 phytoplankton + Rainfall	Top 5 Phytoplankton	0.000	Yes	-76.6
*Model 1.2 Top 5 phytoplankton + Rainfall	Rainfall	0.000	No	-117
Model 2: Days closed + Top 5 Phytoplankton + Water temperature	Days Closed	0.102	No	NA
Model 2: Days Closed + Top 5 Phytoplankton + Water temperature	Water temperature	0.000	Yes	NA
Model 2: Days Closed + Top 5 Phytoplankton + Water temperature	Top 5 Phytoplankton	0.000	Yes	NA
Model 2.1: Top 5 Phytoplankton + Water temperature	Water temperature	0.000	Yes	-12.34
Model 2.1: Top 5 Phytoplankton + Water temperature	Top 5 Phytoplankton	0.000	Yes	11.6
*Model 2.2: Days closed + Water temperature	Water temperature	0.000	Yes	-31.01
**Model 2.2: Days closed + Water temperature	Days Closed	0.514	No	-61.70
*Model 2.3: Days Closed + top 5 phytoplankton	Days closed	0.000	No	-289.9
*Model 2.4: Days Closed + Top 5 phytoplankton	Top 5 phytoplankton	0.000	Yes	-101.35

The final multivariable model retained water temperature in it, as it was deemed to be a confounder, and the presence of any of the top 5 phytoplankton species in trigger levels was a significant explanatory variable to help explain the prevalence of OsHV-1.

Discussion – Data Analysis

In this analysis we examined the relationship between factors that were hypothesized to be risk factors for OsHV-1 manifesting as disease. We already knew that the aetiological agent for OsHV-1 is the virus itself, however it has been suggested that co-infection with *Vibrio* spp. or certain environmental factors may be risk factors for increasing susceptibility to OsHV-1 manifesting as disease. Therefore we were examining relationships between the chosen factors and the presence of OsHV-1 to see if there was any association or correlations present. This is not assigning causation, but if one particular factor was showing a strong association, more effort might be directed to examining this factor more closely. In the marine environment, many environmental factors are subject to small scale variation which may be important in determining these associations, so when grosser scale data are used, relationships are likely to be less obvious. In particular very specific small scale spatial variability has been demonstrated by Paul-Pont (2013).

Average prevalence of OsHV-1 in Croisilles Harbour was low. Only a few of the sampling events showed the presence of OsHV-1, and the prevalence was under 5% until February 2018. Where the virus was present the Ct values were usually recorded as being close to 30 or 40, indicating that even when the virus is present it is present in low quantities. More frequent sampling may have shown different results. Runt oysters appeared to have a higher prevalence of OsHV-1 in general, but the Ct scores were higher (lower viral quantities). This could be because small oysters (runts) could be stunted in growth because they are survivors of the disease, and therefore still carry residual virus in the tissue. There is a negative correlation between growth rate and mortality, however, culling small oysters may change the genetic profile meaning that fast growing oysters which are generally selected for, may be more susceptible to disease (Taris et al., 2006). In this case the oyster culling occurs at the hatchery, but once the spat was put out, no further culling occurred, only grading. Observations from the oyster farmer suggested that during mortality events it was often the larger, or upgraded, spat that died, which does seem to agree with the observation of negative correlation with growth rate and mortality. One could postulate that the faster growing oysters might have expended a lot of energy on growth and therefore were immunocompromised and more likely to die due to OsHV-1 infection.

Water temperature is well known as a risk factor for OsHV-1, especially when there is a rapid increase (Alfaro et al., 2018, de Lorgeril et al., 2018). Certain water temperature ranges appear to be associated with differing levels of risk of a mass mortality (with a range in the 15-17 degree range being the greatest risk) due to infection with OsHV-1 (Alfaro et al., 2018). This study confirms those findings. The greatest spike in temperature in February 2018, coincided with the highest infection of OsHV-1. This followed a mortality event that had occurred the previous December (Aaron Panell, Pers comm). OsHV-1 is known as a disease to which juvenile oysters are susceptible, so the high prevalence in juvenile oysters in February following this mortality is unsurprising, and at this time the lowest Ct values were also recorded (indicating higher viral load). It is possible that these juvenile oysters that have survived this outbreak may now be affected in terms of slower growth rates (Taris et al., 2006). Water temperature was found to be a confounder in the logistic regression models indicating that increased water temperature could be on the causal pathway for OsHV-1 related mortalities to occur, and that viral load or presence alone as a causal agent cannot be considered in isolation without taking into account the water temperature.

It has been suggested that for OsHV-1 to manifest as disease there needs to be a certain amount of virus and co-infection with one of several *Vibrio* spp (Lorgeril et al., 2018, Alfaro et al., 2018). The ability to analyse co-infection of OsHV-1 with presence of *Vibrio* spp. was limited due to the very small number of positive results both of OsHV-1 and of *Vibrio* spp. In only one case did an oyster present with both OsHV-1 and one of five of the target of *Vibrio* spp. So even though these data were analysed, any results should be interpreted with caution. Therefore this study was unable to elucidate further on this point.

Other environmental data that were collected and analysed as potential risk factors showed some interesting results. Rainfall when measured in either total amount fallen cumulatively over the month preceding sampling, or how many days the area was closed due to a flush of rainwater (i.e. heavy rainfall over a short period of time) was a significant factor associated with higher prevalence of OsHV-1. The odds of detecting OsHV-1 was increased by 1.004 times for each mm unit of increase in rainfall, or the odds of detecting OsHV-1 was increased by 1.175 times for each additional day of closure due to rainfall. Since the oyster baskets from which these samples were collected are at the water surface, it is possible that the oysters are in a predominantly freshwater layer for a period of time when there is heavy rainwater flushing. This would lead the oysters to remain shut and not feed, causing the oyster to be stressed and immunocompromised making it more likely to become infected by OsHV-1. Water quality has also been postulated to have a significant effect on mortality events given the association with increased stress (and the associated increased susceptibility to pathogens) and additionally salinity changes can affect an oysters immunity leading to mortality (Alfaro et al., 2018). However, this relationship is unclear as some studies suggest that lower salinity due to rainfall, increases mortalities (Costil et al, 2005) whereas Fuhrmann et al (2016) showed higher survival rates at higher salinities. Oyster farmers also suggested turbidity should be examined as a risk factor for increasing susceptibility to OsHV-1. Turbidity or suspended sediments can be associated with mortality and ill thrift of shellfish (Wilber and Clarke, 2000).

Viral particles attach themselves onto particles in the water, to allow a longer in-water survival time and being attached these particles also increases transmission over a slightly larger spatial scale (Paul-Pont et al, 2013, Evans et al, 2015, Alfaro et al, 2018). Potentially this association might have less to do with the plankton itself, but since high levels of phytoplankton in the water means that there are more particles for a viral particle to attach itself to, the relationship with phytoplankton could be related to particle load in the water. Phytoplankton reaching trigger levels means higher particle load in the water.

The presence of any of the phytoplankton *Pseudonitzschia spp., Dinophysis acuta or accuminata, Karenia mikimotoi, Gymnodinium spp. and Alexandrium, spp*. at levels high enough to trigger flesh testing of shellfish show an OR of 4.2, i.e. the odds of detecting OsHV-1 was increased by 4.2 if any of the five species of phytoplankton were present in trigger levels in the month preceding sampling. This indicates a significant effect in explaining increased prevalence or quantity of OsHV-1. One model even suggested it might be a confounding factor which means the levels of phytoplankton should be examined more closely to better determine the relationship of these phytoplankton species to presence of OsHV-1.

It is possible that the relationship to OsHV-1 and other factors were unable to be accurately described because of the small sample sizes of OsHV-1 positive oysters.

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