Technical Guide

Oyster Refrigeration Index: Models to Predict the Growth of *Vibrio parahaemolyticus* and Total Viable Count Bacteria in Pacific Oysters (*Crassostrea gigas*)

Mark L Tamplin
Judith Fernandez Piquer
Tom Ross

AUSTRALIAN SEAFOOD COOPERATIVE RESEARCH CENTRE

Project No. 2007/719

CRC AUSTRALIA

Australian Government
Fisheries Research and Development Corporation

1. Introduction

1.1. What is Vibrio parahaemolyticus?

Vibrio parahaemolyticus is a natural bacterium found in seawater environments around the world. Not all bacteria in this species are the same, and we refer to different types of *V. parahaemolyticus* as 'strains'. The majority of *V. parahaemolyticus* strains do not present a hazard to humans. However, some can produce substances called 'virulence factors' that allow them to cause infections in humans; these strains are referred to as 'pathogenic' strains.

The most well recognised virulence factor in *V. parahaemolyticus* is the 'thermal direct hemolysin'. We commonly refer to these strains as 'TDH' or 'TDH-positive' strains. TDH is a protein which is able to damage cell membranes such as those that line the human intestine. The resulting illness is mild to severe diarrhoea.

Normally, a person must ingest thousands to millions of TDH-positive *V. parahaemolyticus* bacteria before they will become ill.

1.2. How do people become infected with V. parahaemolyticus?

Eating raw oysters that contain TDH-positive *V. parahaemolyticus* is the most common way that people become infected. Oysters are filter-feeders and obtain food, such as algae, by pumping seawater through their body. During this filtering process, bacteria present in the seawater and attached to algae are concentrated in oyster tissues.

As mentioned before, strains (TDH-positive) that cause illness are not normally present at high levels in oysters at the time of harvest. Instead, human disease is caused when *V. parahaemolyticus* replicates in oysters when they are held at high storage temperatures. If oysters are kept un-refrigerated for an extended period of time, TDH-positive strains can reach levels that may cause a person to become ill if the oysters are eaten raw.

1.3. How can the growth of V. parahaemolyticus in oyster be controlled?

Like most pathogenic bacteria, *V. parahaemolyticus* does not grow when food is properly refrigerated. As the temperature of food increases, *V. parahaemolyticus* progressively grows faster.

Another factor that much be considered when trying to understand and control *V. parahaemolyticus* growth, is the species of oyster. Also, unlike most foods, raw shellstock oysters are alive just before they are eaten. Each live oyster species has a unique immune system that allows it to control bacteria that naturally colonise oyster tissues. Some oyster species may have stronger defences against *V. parahaemolyticus* than other species. Understanding differences between oyster species is one aspect of the research that led to the *Oyster Refrigeration Index* model.

1.4. What is a Predictive Model?

As mentioned above, temperature is a key factor to control *V. parahaemolyticus* growth in oysters. However, research is required to understand how the *V. parahaemolyticus* growth rate changes as the temperature changes. Once this information is known, the data can be converted into a *mathematical model* that will *predict* the growth of *V. parahaemolyticus* in oysters as a function of temperature.

Mathematical models are not easy for everyone to use. Consequently, a user-friendly interface needs to be produced that will allow for simple use and

interpretation of predictions. Further in this guide, you will find specific information about how to use the *Oyster Refrigeration Index* models.

1.5. How can predictive models be useful tools for the oyster industry?

Most companies would agree that it is important to know how the quality and safety of oysters is affected by factors in the supply chain, especially for conditions that the company has little or no control over after the oysters leave the farm.

The temperature profile of oysters in the supply chain has a major effect on product quality (e.g. shelf-life) and safety (e.g. *V. parahaemolyticus*). Predictive models allow you know how quality and safety are influenced by temperature, either by directly examining temperature profiles (e.g. temperature data loggers) or looking at potential scenarios in the cold chain. This information reduces your uncertainty about the product <u>and</u> increases the confidence of health officials in your operations.

For example, your product might be exported to an overseas market where import inspectors have set maximum limits for *V. parahaemolyticus*. The *Oyster Refrigeration Index* allows you to look at “what if?” scenarios, and to design and monitor cold chains.

In another example, you could use the model to predict Total Viable Count (TVC) to estimate shelf-life and potential product loss, and to analyse the performance of your supply chain for maintaining quality.

1.6. How was the Oyster Refrigeration Index developed?

The *Oyster Refrigeration Index* was produced by injecting Tasmanian Pacific oysters with a mixture of *V. parahaemolyticus* pathogenic and environmental strains, and then measuring the growth rate of total *V. parahaemolyticus* in oysters stored from 4 to 30°C. Next, a mathematical (secondary) model was used to describe the change in *V. parahaemolyticus* growth rate as a function of storage temperature. The model predictions were validated against Pacific oysters harvested in New South Wales, which contained natural populations of *V. parahaemolyticus*.

Next, we produced two model interfaces (Excel® and Internet versions) and then evaluated their use with oyster companies. Based on these evaluations, the model interfaces were improved to enhance both format and interpretation.

2. Excel® model interface

2.1. Introduction

This software program is based on experimental data produced at the University of Tasmania Food Safety Centre from Seafood CRC projects 2007/719 and 2008/700.

The software includes a number of 'checks' to avoid errors being made, and you may see error messages arise. They are usually self-explanatory.

Most of the cells in the spreadsheet are protected; however you can enter your data in the designated places. When you have finished using the software, simply save and close the window. The values that were entered in the data input spaces will be there when you next open the spreadsheet.

There are two versions: 1) using $\log_{10}$ values and 2) non-log values.

2.2. General layout

2.2.1. Screen fit

We suggest that you adjust the view so that you can visualise cells for as much of the input and output areas as possible.

2.2.2. Layout

There are six (6) sheets in the Excel® workbook; 'Start Here', 'Manual Input', 'Scenario Design', 'Logger Input', 'Printout' and 'Instructions'. More details are provided below.

2.2.2.1. "Start Here"

You **must** start with this sheet to set initial values for *V. parahaemolyticus* and Total Viable Counts, including maximum limits for each. The sheet is linked to each of the three (3) model formats.

2.2.2.2. "Manual Input"

The sheet allows the user to enter a series of storage times and corresponding temperatures.

2.2.2.3. "Scenario Design"

This model format makes predictions based on time and temperature profiles at specific points in a supply chain.

2.2.2.4. "Logger Input"

Time-temperature profiles from data loggers can be pasted into the spreadsheet to predict *V. parahaemolyticus* growth.

2.2.2.5. "Printout"

This sheet allows you to see and print the data generated from the 'Logger Input' worksheet.

2.2.2.6. "Instructions"

The sheet is a technical guide that explains how the models were developed, and how to use each of the model formats.

2.3. Model formats

2.3.1. "Start Here"

2.3.1.1. Set the initial *V. parahaemolyticus* level in oysters.

The initial level of *V. parahaemolyticus* in oysters can be set in one of three ways: 1) the predicted level based on seawater temperature (recommended), 2) entering a known value, or 3) leaving both cells blank resulting in a default value of 1 cell/g (0 log CFU/g).

2.3.1.2. Set the maximum tolerance level for *V. parahaemolyticus* in oysters.

The tolerance level for *V. parahaemolyticus* is set from a drop-down menu.

2.3.1.3. Set the initial Total Viable Count (TVC) level in oysters.

The initial level of Total Viable Count (TVC) can be set in two ways: 1) leave the cell blank (recommended) to generate a value of 5 log CFU/g, or enter a specific value.

2.3.1.4. Set the maximum Total Viable Count (TVC) level in oysters.

The level of Total Viable Count that indicates the end of oyster shelf-life can be set two ways: leave the cell blank (recommended) generating a value of 7.9 log CFU/g, or set a value using the drop-down menu.

2.3.2. "Manual Input"

This sheet allows the user to input a sequential list of storage times and temperatures, and then to see how the storage conditions affects the predicted levels of *V. parahaemolyticus* and TVC in Pacific oysters.

2.3.2.1. Inputs

You cannot change these values on this sheet because the initial *V. parahaemolyticus* and TVC levels have already been set using the "Start Here" sheet.

You only need to enter oyster storage times and temperatures. Storage times are sequential values from the time of harvest, not a duration at each step (the "Scenario Design" sheet should be used for duration of time for stages in the cold chain).

2.3.2.2. Outputs

The predicted changes in *V. parahaemolyticus* and TVC levels are shown in the orange area.

2.3.2.2.1. Graph

The initial, net change and final levels of *V. parahaemolyticus* and TVC are shown above the graph.

Plotted on the graph are the changes in *V. parahaemolyticus* and TVC levels, storage temperature, and two horizontal lines: the maximum limit of acceptability for *V. parahaemolyticus* levels (set in the Start Here sheet) and the maximum level of TVC (set in the Start Here sheet). The horizontal axis is time in days since harvest. The left vertical axis is the level of *V. parahaemolyticus* and TVC in log CFU/g). The right vertical axis is temperature in degree C.

2.3.3. "Scenario Design"

This model format is useful to examine changes in predicted levels of *V. parahaemolyticus* and TVC in Pacific Oysters at specific stages in the supply chain. It can also help you identify stages in the chain where conditions permit *V. parahaemolyticus* and TVC to grow, and where better controls may be needed.

2.3.3.1. Inputs

The initial *V. parahaemolyticus* and TVC levels have already been set using the "Start Here" sheet and cannot be changed.

In each row of the table on the bottom left side, you need to type in the name of the stage, the <u>duration of time</u> at the stage, and the temperature at the stage. (**When you type in a new row, the row below it will change from grey-shaded boxes to white boxes**.)

The temperature at each stage should be the anticipated average temperature because the starting temperature will likely be different than the final temperature.

2.3.3.2. Outputs

The predicted changes in *V. parahaemolyticus* and TVC levels are shown in the orange area on the right side of the sheet.

2.3.3.2.1. Table

Rows in the table under the graph correspond to the input rows on the left side of the sheet. Cells show the predicted change in levels at each combination of time and temperature, and the total level of bacteria at that stage.

On the top of the table are six (6) cells that show the initial, net change in, and final *V. parahaemolyticus* and TVC levels over the entire storage time scenario.

2.3.3.2.2. Graph

Plotted on the graph are the changes in *V. parahaemolyticus* and TVC levels, storage temperature, and two horizontal lines: a maximum acceptable level for *V. parahaemolyticus* (set in the Start Here sheet) and a maximum acceptable level of TVC (set in the Start Here sheet). The horizontal axis is time in days since harvest. The left vertical axis is the level of *V. parahaemolyticus* and TVC in log CFU/g). The right vertical axis is temperature.

2.3.4. "Logger Input"

This model format is designed for companies using temperature data loggers. Where you place the data loggers in oyster pallets can have a large significant effect on predictions of bacterial growth and should be carefully considered. For the most conservative estimates of cooling, place the logger in the centre of the sack or in a sack located in the centre of the pallet. As for the "Scenario Design" version, this version can also help you identify stages in the cold chain where conditions permit *V. parahaemolyticus* and TVC to grow fastest, and where better controls are needed to increase shelf-life and safety.

2.3.4.1. Inputs

The initial *V. parahaemolyticus* and TVC levels have already been set using the "Start Here" sheet.

After you have downloaded your logger data, enter the start date and start time.

In the next input row below, enter the logger time interval **in minutes**. The data in the grey-shaded area should be exactly the same as your logger data.

Finally, cut and paste the temperatures from your data logger into the column to the right of the grey-shaded area. As mentioned above, the data should be the same as your logger data.

2.3.4.2. Outputs

The predicted changes in *V. parahaemolyticus* and TVC levels are shown in the orange area on the right side of the sheet.

Above the graph are the initial, net change in, and finals levels for *V. parahaemolyticus* and TVC.

2.3.4.2.1. Graph

Plotted on the graph are the changes in *V. parahaemolyticus* and TVC levels, storage temperature, and two horizontal lines: a maximum limit for *V. parahaemolyticus* levels (set in the Start Here sheet) and the maximum level of TVC (set in the Start Here sheet). The horizontal axis is the date and time since harvest. The left vertical axis is the level of *V. parahaemolyticus* and TVC in log CFU/g). The right vertical axis is temperature.

2.3.5. Printing and the "Printout' sheet

To print the results from the various sheets, simply use the normal 'Print' command on your computer. The print area has been set to show all inputs and results, to print in 'landscape' format and to fit the print area to a single page. Depending on your printer however, you may need to adjust the printer settings so that it is all printed on a single page.
The results of the "Logger Input" sheet are also shown and can be printed from the "Printout" sheet.

2.3.6. "Instructions" sheet

Technical instructions are located in this sheet.

3. Internet model interface

3.1. General layout

Input cells are on the left side and top of the page, and output (Results) on the right. Error codes are located at the bottom left side of the page.
Buttons to manage your account, switch between log and non-log versions and to login or logout are located just under the title at the top.

3.2. Creating an account

The login page has links to request an account (where you will get the email to activate) and a password reminder page.
You can use the Manage Account button to change your profile information, including your password. When you edit your information, you will need to enter your password.

3.3. Initial setup

Step 1 – select the $\log_{10}$ or non-$\log_{10}$ preference
You have the choice of using the tool as a log or non-log version. This affects both how you input the levels of *V. parahaemolyticus* and TVC, and the output.
The default version of the non-log version. To change to the log version, click on the button "Switch to log scale."

Step 2 – select the input format
Using the drop-down menu next to "Input Method", select either the "Manual input (time in hours)", "scenario design (duration in hours)" or "data logger (date/time)" version. See below for more detailed explanation about the features for each of these versions.
If you switch between these formats, your input data will be reset to sample values, so please select the appropriate format before inputting your data.

Step 3 – select the method for calculating temperature
The format for calculating temperature for each step in the storage profile can be set in two ways:

- "constant for each step" – this assumes that the temperature of the oysters is immediately at the temperature that you specify.
- "average between steps" – this assumes that the temperature will change (increase or decrease) linearly to the temperature for this step from the temperature in the previous step. The tool will linearly interpolate the change in temperature over ten intervals of equal time.

Step 4 - set the initial level of *V. parahaemolyticus* in oysters in one of two ways:

- the predicted level based on harvest seawater surface temperature (recommended), or
- enter a specified value in cells per gram or log CFU/g (depending on whether you use the log or non-log version

Click the button (circle) for the preferred method, and then enter either the seawater surface temperature or the initial level on a per gram basis.

Step 5 - set the initial TVC level in one of two ways:

- leave the cell blank to generate a start level of 100,000 CFU per gram (log 5 CFU/g). This is a common level for freshly harvested oysters. Or,
- enter a specified level per gram

Step 6 – set an upper limit for *V. parahaemolyticus* and TVC using the drop-down menu. These can be arbitrary levels or those that may be set by a regulatory body or customer.

Step 7 – calculate the growth of *V. parahaemolyticus* and TVC
Finally, click the "Compute Growth" button. The output will appear in the yellow box. To the immediate left of the graph you will also see the predicted level for each stage (row).

3.4. Model versions

3.4.1. "Manual input (time-in-hours)"

3.4.1.1. Entering time and temperature

For this version, it is optional to enter information in the column labelled "Stage".

Add your oyster storage time and temperature information. Storage times are sequential values from the time of harvest, not duration at each step (the "scenario design" version should be used for duration of time for each operation in the cold chain).

3.4.1.2. Adding more rows

If you need to add or remove rows, enter the number of rows in the cell to the right of "Number of rows" and then click "Add Rows" or "Remove Rows".

3.4.1.3. Computing results

Finally, click the "Compute Growth" button. It may take a some seconds for the screen to refresh.

3.4.1.4. Output (Results)

3.4.1.4.1. Table

Above the graph are shown the initial, predicted change and finals levels for *V. parahaemolyticus* and TVC.

3.4.1.4.2. Graph

Plotted on the graph are the changes in *V. parahaemolyticus* and TVC levels, storage temperature, and the maximum limit for *V. parahaemolyticus* and TVC levels that you previously set.

The horizontal axis is time (days) since harvest. The left vertical axis is the level of *V. parahaemolyticus* and TVC. The right vertical axis is temperature.

3.4.2. "Scenario design (duration in hours)"

3.4.2.1. Entering time and temperature

Enter information in the column labelled "Stage". Examples are provided in the default setting.

Add oyster storage time and temperature information as described for the "Manual input" version, above.

Note - storage times are duration in hours, not the time from harvest.

3.4.2.2. Adding more rows

Same as "Manual input" version, above.

3.4.2.3. Computing results

Click the "Compute Growth" button. It may take a some seconds for the screen to refresh.

3.4.2.4. Output (Results)

3.4.2.4.1. Table

Same as "Manual input" version, above.

3.4.2.4.2. Graph

Same as "Manual input" version, above.

3.4.3. "Data logger"

3.4.3.1. Entering date and time and temperature

You can manually enter data in the two columns or upload a file.

To see an example of how to format a CSV file, click on "Sample Data Logger File".

Sample data are provided in the default setting.

3.4.3.2. Adding more rows

Same as for "Manual input" version, above.

3.4.3.3. Computing results

Click the "Compute Growth" button. It may take a some seconds for the screen to refresh.

3.4.3.4. Output (Results)

3.4.3.4.1. Table

Same as for "Manual input" version, above.

3.4.3.4.2. Graph

Same as for "Manual input" version, above.