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Viability PCR for aquatic animal pathogens

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1 Executive Summary

In 2014, commercial aquaculture generated an estimated $500 million for the New Zealand economy and employed more than 3000 people (C. Johnston, pers. comm.; http://aquaculture.org.nz/industry/overview/). This industry aims to generate $1 billion per annum by 2025 (Ministry for Primary Industries (MPI), 2015). Geographical isolation and border controls have kept New Zealand relatively free from many of the pathogens that impact aquaculture production elsewhere in the world (e.g., http://www.oie.int/international-standard-setting/aquatic-code/access-online/). However, the introduction, exacerbation or spread of pests and pathogens remains an ongoing threat to New Zealand's aquaculture, fisheries and environment (Georgiades et al., 2016). These can lead to losses in production, increased production costs and potential impacts to trade, tourism and environmental and socio-cultural values.

Recently, New Zealand's aquaculture industry has been impacted by the occurrence of significant disease incidences (e.g., *Bonamia ostreae* in oysters; Lane et al., 2016, New Zealand rickettsia-like organism (NZRLO), and *Tenacibaculum maritimum* in salmon; Brosnahan et al., 2017). In circumstances such as these, the delivery of timely diagnostic information is key to informing management decisions to prevent pathogen spread. Traditionally, the identification of viable (live) pathogens in the aquatic environment is time-consuming and complicated by the possible presence of viable but non-culturable (VBNC) organisms, leading to the reporting of false negative results. The optimisation, validation and uptake of molecular diagnostic tools to discriminate between viable and non-viable target organisms would provide decision makers, both within MPI and the aquaculture industry, with more timely information from which to make decisions.

Polymerase chain reaction (PCR) of nucleic acids is one of the most common tools used by diagnostic laboratories for pathogen detection due to its specificity, sensitivity and efficiency (OIE, 2016). However, PCR results do not give the end user any information about target organism viability i.e., is it living and potentially infectious. The advent and adoption of new technologies that overcome this issue (i.e., viable PCR (vPCR)) has the potential for many applications within MPI including biosecurity (pre and post-border diagnostics), market access and food safety. To date, this technology has been applied to a number of different organisms: Gram-negative bacteria (e.g., Barbau-Piednoir et al., 2014, Liu et al., 2014, Duarte et al., 2015, Hess et al., 2015), Gram-positive bacteria (e.g., Rueckert et al., 2005, Kramer et al., 2009, Cattani et al., 2013, de Assuncao et al., 2014), viruses (Fittipaldi et al., 2010, Graiver et al., 2010, Parshionikar et al., 2010, Fongaro et al., 2016), fungi, yeasts and oomycetes (e.g., Rawsthorne et al., 2009, Agullo-Barcelo et al., 2013, Agusti et al., 2013), amoeba (Fittipaldi et al., 2011), nematodes (Christoforou et al., 2014) and protozoa (Habtewold et al., 2015).

Viability PCR has had limited application in the aquatic space with four known studies in shellfish and crustaceans (Mamlouk et al., 2012, Zhu et al., 2012, Moreau et al., 2015, Moreno et al., 2015) and three in finfish (Lee et al., 2006, Mamlouk et al., 2012, Maće et al., 2013). Improvement and validation of diagnostic tools for early detection of aquatic pathogens, including tools that can determine pathogen viability, is important to New Zealand's growing aquaculture industry and the protection of aquatic environments. For example, this industry has indicated their preference for the preventive use of

vaccines rather than to reactively treat diseases with antibiotics (Funding for Salmon Research, 2013). In 2017, the first vaccine for New Zealand aquaculture was approved for use on NZ-RLO in salmon (MPI, pers. comm.). This vaccine is manufactured using dead *Piscirickettsia salmonis* from Chile, an exotic organism to New Zealand. This may pose a challenge for diagnostic laboratories as dead *P. salmonis* DNA may be detected by PCR from vaccinated fish. Having tools that can quickly differentiate between the DNA of vaccinated fish and live pathogens in a fish is therefore required.

A recent "proof of concept" project at MPI's Plant Health and Environment Laboratory (PHEL) (funded by the MPI Operational Research Programme) demonstrated that vPCR technology was able to detect live phytopathogenic bacteria (*Pseudomonas syringae* pv. *actinidiae*) and differentiate between viable and non-viable cells in kiwifruit leaf tissue and pollen (Taylor et al., 2014). Because of the potential benefits across MPI's diagnostic services, this technology was transferred to the Animal Health Laboratory (AHL) and an Operational Research project funded to optimise and validate it on model aquatic animal pathogens.

The objectives for this project were to:

- Review the scientific literature, outline the experimental design, and evaluate basic testing protocols for the application of vPCR to aquatic animal pathogens.
- Optimise and validate vPCR assays for the detection of model bacterial and viral aquatic animal pathogens.
- Develop vPCR testing protocols to allow for the detection of viable (live) pathogens in salmon kidney, liver and skin tissue.
- Carry out inter-laboratory testing of the developed vPCR protocols to assess assay reproducibility and repeatability, as possible.
- Assess the feasibility of, and validate, a high-throughput vPCR protocol for target organisms within salmon tissue.
- Develop a protocol for the application and interpretation of each assay and "how to" guidelines for validation of a new matrix or target organism.

By accomplishing the above objectives, this work has filled key knowledge gaps regarding the optimisation and application of this technology for aquatic animal bacteria and viruses, and provided operational protocols for the two bacterial species in salmon tissues.

It is anticipated that vPCR will be an immediately valuable resource for MPI for determining pathogen viability particularly when vaccines are used by New Zealand's aquaculture industry.

This study also provided initial optimisation and validation of a method for transferring this technology to a terrestrial pathogen (*Mycoplasma bovis*) and a different matrix (bull semen) (Appendix 6).

2 Literature review

This review outlines the current methods used for detecting viable organisms within diagnostic samples and includes the experimental parameters considered necessary for optimisation and validation of the vPCR technology. The model fish pathogens proposed for use during this project are discussed along with the rationale for their selection.

2.1 METHODS TO DETERMINE VIABILITY

Traditional culture techniques (i.e., plating, broth culture, tissue culture, etc), are the most commonly used diagnostic tools to detect viable pathogens in a tissue sample. However, these methods can be time consuming and have the potential to produce false negative results depending on the target organism. This can be due to the organism's fastidious nature, overgrowth by faster growing environmental organisms in the sample, poor sample quality, incorrect nutrient media or cell line used, or organisms being in a viable but non-culturable state. Fastidious bacterial species, which can include target organisms, require growth on specialised media for long periods, for example, *Mycobacterium* species can take up to four weeks before a diagnosis is possible (Pfyffer et al., 2012). Similarly, for organism types where cell lines exist, a standard virus isolation test using cell culture takes a minimum of two weeks, while four weeks is recommended (LaPatra 2014). Such time constraints to confirm results during a biosecurity response or for export testing can diminish the management options available and negatively impact the outcomes. Due to these difficulties, molecular methods have been employed in an attempt to assess organism viability.

The most common molecular method used to determine pathogen viability is based on targeting ribonucleic acid (RNA) (Josephson et al., 1993). More recently a vPCR technique using modifying dyes to intercalate[1] with nucleic acid of non-viable cells has been developed (Nogva et al., 2003, Nocker et al., 2009).

RNA is used as a marker for cell viability for two reasons: 1) messenger RNA is only produced by metabolically active cells; 2) RNA is more unstable and degrades more rapidly after cell death compared to deoxyribonucleic acid (DNA). For example, DNA has been shown to remain stable in the environment for up to three weeks after the death of an organism (Nocker et al., 2006) or for up to 55 days in seawater (Nocker et al., 2010). While RNA appears to be a good candidate for assessing viability, there are still some drawbacks of associating a positive RNA signal with viability, including:

- RNA is difficult to extract in high quality due to its instability (Andorra et al., 2010).
- The requirement of a pre-treatment of nucleases to the sample prior to RNA extraction to ensure only RNA within live cells is extracted and detected by PCR. This may not be applicable for all types of RNA viruses (Nuanualsuwan et al., 2002).

[1] The inclusion of the dyes into the nucleic acid structure.

- RNA extraction is prone to contamination with RNA-degrading enzymes generating results that are not reproducible, especially from difficult matrices (Desneux et al., 2015).
- The amount and stability of RNA is not consistent, with some RNA molecules able to persist for up to 30 hours after loss of cell viability. This is dependent on factors such as environmental conditions and the region of the genome that is amplified (Birch et al., 2001).
- When slow-growing or dormant cells are present, it is likely the RNA content will be below the limit of detection for PCR even though the cells are still live (Fittipaldi et al., 2012).

The use of dyes to determine cell viability by staining various parts of the target organism prior to microscopic examination has been in laboratory use for many years, for example: trypan blue; neutral red; crystal violet; SYTO9; hexidium; SYTO59; and malachite green (DeRenzis et al., 1973, Pourcho et al., 1978, Ishiyama et al., 1996, Belosevic et al., 1997, Hauton et al., 1998). However, these dyes do not lend themselves to high-throughput sample processing or high specificity of the target organism in a mixed sample.

A more advanced use for the application of dye technology is vPCR, the use of nucleic acid intercalating dyes coupled with PCR. This technique was first described by Nogva et al., (2003) using ethidium monoazide (EMA). Since then, propidium monoazide (PMA) and PEMAX (a mixture of EMA and PMA) have been investigated to improve the assay efficiency.

EMA, PMA and PEMAX are able to enter cells with compromised membranes (i.e., dead cells) and intercalate with the nucleic acids present. The bound molecule is then exposed to light of a certain wavelength (446-474 nm) which crosslinks the nucleic acid thereby inhibiting amplification by PCR.

A reported limitation of EMA is that it is able to cross the membranes of some live cells (Nocker et al., 2006). However, it was thought that metabolically active cells would expel EMA into the surrounding media as they do with ethidium bromide, the dye it is derived from (Codony et al., 2015).

PMA is based on propidium iodide, a common membrane impermeant dye that has been used extensively in live-dead determination by flow cytometry and fluorescent microscopy (Nocker et al., 2006). PMA was designed to overcome the limitation of EMA, however PMA will not penetrate cells that are dead but still have an intact membrane, for example cells treated with UV. UV is commonly used to eliminate microorganisms from water (e.g., treatment of effluent from land-based aquaculture; ballast water management systems), thus the efficacy of PMA vPCR following such treatment needs to be assessed.

PEMAX, the latest dye to be investigated, is a mixture of PMA with lower concentrations of EMA; approximately < 10 µM EMA and ≥ 20 µM PMA (Codony et al., 2015, Agusti et al., 2017, Daranas et al., 2018). This dye is able to intercalate with nucleic acid of dead cells with intact membranes which lack the metabolism to inhibit its uptake (Nocker et al., 2009, Cangelosi et al., 2014, Codony et al., 2015).

2.2 EXPERIMENTAL DESIGN REQUIREMENTS

vPCR has many variables and parameters, therefore the experimental design for optimisation and validation requires careful consideration to ensure the outcomes are statistically robust and repeatable. It is apparent that no “one size fits all” method can be applied as many of the parameters are likely to differ depending on the target pathogen, matrix applied and PCR assay used (Nocker et al., 2006, Codony et al., 2015).

The key parameters to be optimised for this project include:

- Concentration of pathogen and inactivation protocol.
- Type of dye (i.e., EMA, PMA, PEMAX).
- Concentration of dye.
- Incubation time of sample with dye.
- Temperature of incubation with dye.
- Photoperiod of sample (i.e., photoactivation system for tubes (PhAST) or photoactivation universal light (PAUL)).
- Resuspension buffers to increase effectiveness of dye penetration, e.g., sodium deoxycholate (SD), surfactants (e.g., Triton X-100 and Span 20), reaction buffer+, pH levels, and nutrients.
- Detection of the pathogen in mixed bacterial populations.
- Effects of sample matrix.
- Target PCR amplicon length.

It is suggested that the performance of EMA, PMA and PEMAX is specific for organism type. PMA has been suggested to be more effective than EMA on bacterial cells and spores (Rawsthorne et al., 2009, Agusti et al., 2013, Kruger et al., 2014). More recent vPCR studies typically use PEMAX (Agusti et al., 2017, Thanh et al., 2017, Lizana et al., 2017). Codony et al. (2015) demonstrated that PEMAX was optimal for bacteria. PMA and EMA have both been used on viral particles with varying success (Graiver et al., 2010, Parshionikar et al., 2010, Karim et al., 2015). The one study comparing PEMAX to PMA found that PEMAX was more effective for norovirus (Randazzo et al., 2016). EMA and PEMAX were assessed in the present study to compare the old and new technology. PMA was considered as a backup if results from these two dyes were not sufficient.

Dye concentration is important for reducing false negative and false positive results. Too much dye could be cytotoxic and penetrate live cells while too little could reduce the effective penetration of dead cells. This is particularly true in a natural sample where the dye can bind with other nucleic acids and debris in addition to the target pathogen. Previously investigated EMA concentrations have ranged from 3 to 240 µM (Nocker et al., 2006, Rawsthorne et al., 2009, Andorra et al., 2010, Agusti et al., 2013). PEMAX concentrations commonly range from 50 to 100 µM (Thanh et al., 2017, Daranas et al., 2018). To combat dye toxicity, Takahashi et al., (2017) reduced the initial dye dose by using two separate 50 µM doses. Using a double dose of dye with the addition of a buffer (sodium dodecyl sulfate) resulted in a further differentiation between live and dead cells.

Incubation times and temperatures allowed for the dye to penetrate dead cells have ranged from 5 to 60 min at 0 to 40 °C, respectively (Chang et al., 2009, Rawsthorne et al., 2009, Nkuipou-Kenfack et al., 2013). While 15 min at room temperature (RT) is typically used, these parameters can differ depending on the target organism and the matrix.

The default setting of the PhAST or PAUL light system is 15 min (Barbau-Piednoir et al., 2014, Codony et al., 2015, Duart et al., 2015). However, in some studies optimised photoperiods have improved results (Cattani et al., 2013, Kim et al., 2014, Sangsanont et al., 2014, Desneux et al., 2015). In addition, agitation of the samples during incubation may facilitate nucleic acid to dye exposure and light penetration of dense or coloured matrices (Nkuipou-Kenfack et al., 2013, Sanchez et al., 2014). Agusti et al., (2017) improved differentiation between live and dead cells by exposing samples to a double light treatment coupled with a double tube change.

Resuspension buffers can influence dye penetration into the dead cells thus improving differentiation. Reported reagents include SD, Triton X-100 and Span 20. SD has been used at various concentrations in multiple studies to reduce the rate of false positives by destroying cell walls and membranes (e.g., 0.01, 0.025 %) (Lee et al., 2009, Takahashi et al., 2017). Nkuipou-Kenfack et al., (2013) found that while the use of SD on Gram-negative bacteria was appropriate, it had questionable suitability for Gram-positive bacteria. Of the surfactants that have been tested to increase dye penetration, only Triton X-100 showed promise when applied to hepatitis A virus (Moreno et al., 2015). The solution used for sample dilution may also influence results as changes in pH or salt concentrations may compromise cell walls or membranes. However, vPCR may be improved by the addition of nutrients and active compounds for facilitating reagent diffusion (Codony et al., 2015).

Sample matrix, pathogen concentration and detection of the target pathogen in a mixed bacterial or viral population can also influence vPCR results. Having a sample matrix that is dense, turbid or dark may impact dye effectiveness and light penetration (Zhu et al., 2012, Desneux et al., 2015). Different approaches for optimisation include: dilution of the matrix, separation of cells from the matrix, an increase in dye concentration, agitation of the samples during dye incubation and light exposure, and filtration (Mamlouk et al., 2012, Zhu et al., 2012, Maće et al., 2013, van Frankenhuyzen et al., 2013, Kim et al., 2014, Desneux et al., 2015, Moreno et al., 2015).

Dilution of the sample matrix may also dilute the target pathogen which could impact test sensitivity. Separation of the target cells from the matrix using homogenisation and centrifugation is a possible alternative, however this will be dependent on how well the cells separate from the matrix without compromising membrane integrity. Maće et al., (2013) found that filtration of a homogenised tissue sample allowed for a larger amount of starting material to be used as the eukaryotic cells and salmon DNA were separated from the bacteria prior to the addition of dye. These strategies have the added benefit of reducing the sample turbidity for increased light penetration. Photoperiod appears less important than dye concentration in turbid matrices whereas the reverse was true as turbidity lessens (Desneux et al., 2015).

The length of the target PCR amplicon has also been shown to influence the vPCR outputs. The longer the amplicon, the greater likelihood of complete suppression of a PCR signal from non-viable cells (Agullo-Barcelo et al., 2013, Martin et al., 2013, Ditommaso et al., 2015). However, a longer amplicon target means a conventional PCR will need to be used. Conventional PCR is generally more time consuming, less specific and less sensitive compared to quantitative PCR (qPCR). Some vPCR have been successful using shorter amplicon lengths of < 120 base pairs (bp) in qPCR (Nocker et al., 2007, Desneux et al., 2015), but it is unlikely that complete suppression will be achieved. Therefore, the interpretation of any qPCR results is an important consideration. As it is unlikely that complete suppression will be seen with v-qPCR, formulas similar to a study by Hess et al (2015) may have to be developed for interpretation.

2.3 MODEL ORGANISMS

Three aquatic animal pathogens were examined for the present study; *Yersinia ruckeri*, *Tenacibaculum maritimum* and Aquabirnavirus. These pathogens were chosen as model candidates as they are representative of a range of pathogens of concern to aquatic animal health in New Zealand; a freshwater bacteria, a saltwater bacteria and a virus (Georgiades et al., 2016). As they are endemic to New Zealand, naturally infected populations are more likely to be sourced to allow more rigorous assay validation.

2.3.1 *Yersinia ruckeri*

Yersinia ruckeri is a Gram-negative bacterium in the family *Enterobacteriacea*. It is the aetiological agent of enteric red mouth disease or Yersiniosis in salmonids and non-salmonids worldwide (Carson et al., 2009). There are four recognised serotypes of this organism with only one serotype (O1b) being endemic to New Zealand (Barnes et al., 2016).

Yersinia ruckeri is one of the pathogens tested for exclusion by AHL for salmon export to Australia for human consumption (MAF, 2000). Currently this testing is carried out using traditional culture methods, however application of vPCR offers the advantages of greater efficiency and sensitivity. This organism will be used as a model Gram-negative bacteria in salmon kidney tissue.

2.3.2 *Tenacibaculum maritimum*

Tenacibaculum maritimum is a Gram-negative bacteria of the family *Cytophaga/Flexibacter*, and is the causative agent of marine flexibacteriosis or tenacibaculosis of wild and farmed marine fish worldwide (Avendano-Herrera et al., 2006).

This organism was detected in skin ulcers of diseased fish during recent salmon mortalities at the top of New Zealand's South Island (Brosnahan et al., 2017) and has since been found to be widely distributed (Brosnahan et al., 2019). *T. maritimum* is fastidious, difficult to culture and is often outcompeted by other organisms resulting in false negative results (Toranzo et al., 2015, Pers. Obs.).

This bacteria will be used as a model for vPCR for assessment of a clumping, halophilic bacteria in salmon skin tissue where a mixed population of bacteria are expected.

2.3.3 Aquabirnavirus

Aquabirnavirus (ABV) is a non-enveloped RNA virus in the family *Birnaviridae* which have been isolated from a range in species in fresh and marine waters (Hill et al., 1995). The type species of this genus is infectious pancreatic necrosis virus (IPNV) which causes acute disease in young salmonids (McColl et al., 2009) and is exotic to New Zealand. ABV is endemic to New Zealand and has been found in healthy wild salmonid populations (Tisdall et al., 1987). This virus will be used as a model for non-enveloped RNA viruses in salmon liver tissue.

3 Methods

3.1 NUCLEIC ACID EXTRACTION

DNA or RNA was extracted directly from the EMA or PEMAX treated samples using Qiagen extraction kits. The extraction methods from pure culture or tissues are briefly outlined below.

For DNA:

Qiagen QIAamp HT kit (typical extraction) method: Tissue lysis buffer (ATL, 180 µL) and 20 µL proteinase K was added to either a pellet or 100 µL of suspension and lysed overnight at 56 °C. After tissue lysis, 100 µL lysis buffer (VXL) was added and the sample heated to 65 °C for 10 min. Lysate was then removed from PC3+ (if applicable), transferred to an S-block and the nucleic acid was extracted on the automated robot in PC2 as per the manufacturers' protocol (with the modification of no top elute).

QIAamp DNA mini kit (occasional extraction) method: ATL (180 µL) and 20 µL proteinase K was added to either the pellet or 100 µL of suspension and lysed overnight at 56 °C. After tissue lysis, 200 µL of lysis buffer (AL) was added and the sample was incubated at 70 °C for 10 min. The sample was then removed from PC3+ (if applicable), and the extraction carried out in PC2 as per the manufacturers' protocol.

For RNA:

Cador pathogen 96 QIAcube HT kit: Lysis buffer (VXL, 100 µL), 20 µL proteinase K and 1 µL carrier RNA were added to the sample (either 100 or 200 µL suspension). This was heated at 65 °C for 10 min in PC3+, removed to PC2 and transferred to a lysis block. The RNA was then extracted on the automated robot as per the manufacturers' protocol (with the modification of no top elute).

3.2 PCR AMPLIFICATION

For *Yersinia ruckeri*, a quantitative PCR (qPCR) assay was used with an amplicon size of 109 bp (Keeling et al., 2012). A conventional assay was initially trialled targeting an amplicon size of 575 bp (Gibello et al., 1999). As the conventional PCR assay did not provide complete suppression it was not used for all experiments (used in optimal dye concentration, incubation time, resuspension buffers, long incubation trial, artificially spiked tissue and naturally infected tissue).

For *Tenacibaculum maritimum*, qPCR and nested conventional PCR were used (Cepeda et al., 2003, Fringueilli et al., 2012) with amplicon sizes of 155 and 400 bp, respectively (primary round of nested PCR is 1500 bp).

For ABV, a conventional PCR was used with an amplicon size of 850 bp (McColl et al., 2009). A qPCR was trialled initially, however the sensitivity was found to be tenfold less than the conventional reverse transcriptase PCR (RT-PCR) (Tapia et al., 2015).

For bacterial pure culture, 1 µL DNA was used in the assay in duplicate. For DNA derived from tissues, two DNA template volumes of 2 and 5 µL, were used. For RNA, 5 µL was used as a template in the assay.

3.3 ASSAY SENSITIVITY

Assay sensitivity was performed using optimal vPCR conditions.

Gram-negative bacteria

The sensitivity of the v-qPCR and vPCR assays were determined by performing a dilution series of live bacteria (10^2 - 10^7 CFU mL^{-1} for *Yersinia ruckeri*, 10^1 - 10^6 CFU mL^{-1} for *Tenacibaculum maritimum*) in a background of a high concentration of dead bacteria (10^7 for *Y. ruckeri*, 10^6 for *T. maritimum*), with and without PEMAX dye treatment. A dilution series of live pathogen in molecular grade nuclease free water (Sigma-Aldrich) without dye was also run. All assays were run in triplicate.

The results from the qPCR assays for *Y. ruckeri* and *T. maritimum* were used to determine the sensitivity of each of the specific assays. Bacterial concentration was plotted against the corresponding cycle threshold (Ct) value to determine the R^2 value. The amplification efficiency was assessed using $E = -1+10^{(-1/slope)}$. The limit of detection (LOD) of the assay was determined to be the lowest bacterial concentration where the target molecules increased during each replication cycle (as shown by the Ct value).

For the conventional *T. maritimum* PCR, the LOD was the lowest dilution at which all replicates amplified.

ABV

A dilution series of live virus (10^2 – 10^7 tissue culture infective dose ($TCID_{50}$)) was evaluated with and without PEMAX dye treatment. RNA extraction was then carried out and the conventional RT-PCR was performed. All assays were run in triplicate. The LOD for this assay was the lowest dilution at which all replicates amplified.

3.4 HEAT KILL TREATMENT OF BACTERIA

Pure cultures of *Yersinia ruckeri* and *Tenacibaculum maritimum* were cultured in broth and incubated at 22 °C:

- *Y. ruckeri*: Brain heart infusion (BHI) for 24 h.
- *T. maritimum*: Tryptone, yeast, glucose media with salt (TYG-M) for 48 h.

Spread plate dilutions of the incubated broth onto suitable agar (*Y. ruckeri* = Columbia sheep's blood agar (BA), *T. maritimum* = TYG-M agar) were performed in triplicate to confirm the CFU mL^{-1}.

Aliquots (1 mL) of each culture were treated at: 37, 56 and 99 °C for 10 min. An aliquot (100 µL) from treated samples was spread plated onto the appropriate agar in triplicate to ensure treatment efficacy.

All agar plates (from live as well as heat treated cultures) were incubated at 22 °C for 7 d to confirm the starting concentration, cell death and the optimal killing temperature.

3.5 HEAT KILL TREATMENT OF VIRUS

A pure culture of ABV was grown up in a medium (75 cm^3) tissue culture flask with a monolayer of Chinook salmon embryo cell line (ChSE-214) for 7 d at 15 °C. The concentration of the virus was determined by carrying out a titration in a 96-well tissue culture plate with a ChSE monolayer in duplicate and incubated for 14 d. Titration was calculated according to the Spearman-Kärber method (Spearman 1908, Kärbe 1931).

Aliquots (1 mL) of culture were initially treated at: 37, 56 and 99 °C for 10 min. An aliquot (100 µL) of the heat killed virus from each temperature was then re-inoculated onto a small (25 cm^3) tissue culture flask with a monolayer of ChSE cells and incubated for at 15 °C for 7 d.

3.6 DYE CONCENTRATION AND INCUBATION TIME

For each treatment, all optimisation experiments were performed in triplicate with separate broth cultures (i.e., independent replicates).

Three dilutions of heat treated (dead) bacteria and virus were performed. The concentrations used for the pathogens were:

- *Yersinia ruckeri* were 10^9, 10^5 and 10^2 CFU mL^{-1}.

- *Tenacibaculum maritimum* 10^8, 10^5 and 10^3 CFU mL^{-1}.
- ABV 10^7, 10^5 and 10^4 $TCID_{50}$.

These dilutions were assessed with each of the following concentrations of dye (Figure 1):

- PEMAX: 10, 25, 50 and 100 µM.
- EMA: 10, 25, 50 and 100 µM.

Samples were incubated in the dark and exposed to PhAST blue light for 15 min.

The following experimental controls were used:

- Dead cells with no dye and no exposure to light.
- Live cells with no dye and no exposure to light.
- Dead cells with no dye and exposure to light.
- Dead cells with dye added (EMA: incubated at RT for 5 min; PEMAX: 22 °C for 30 min) and no exposure to light.

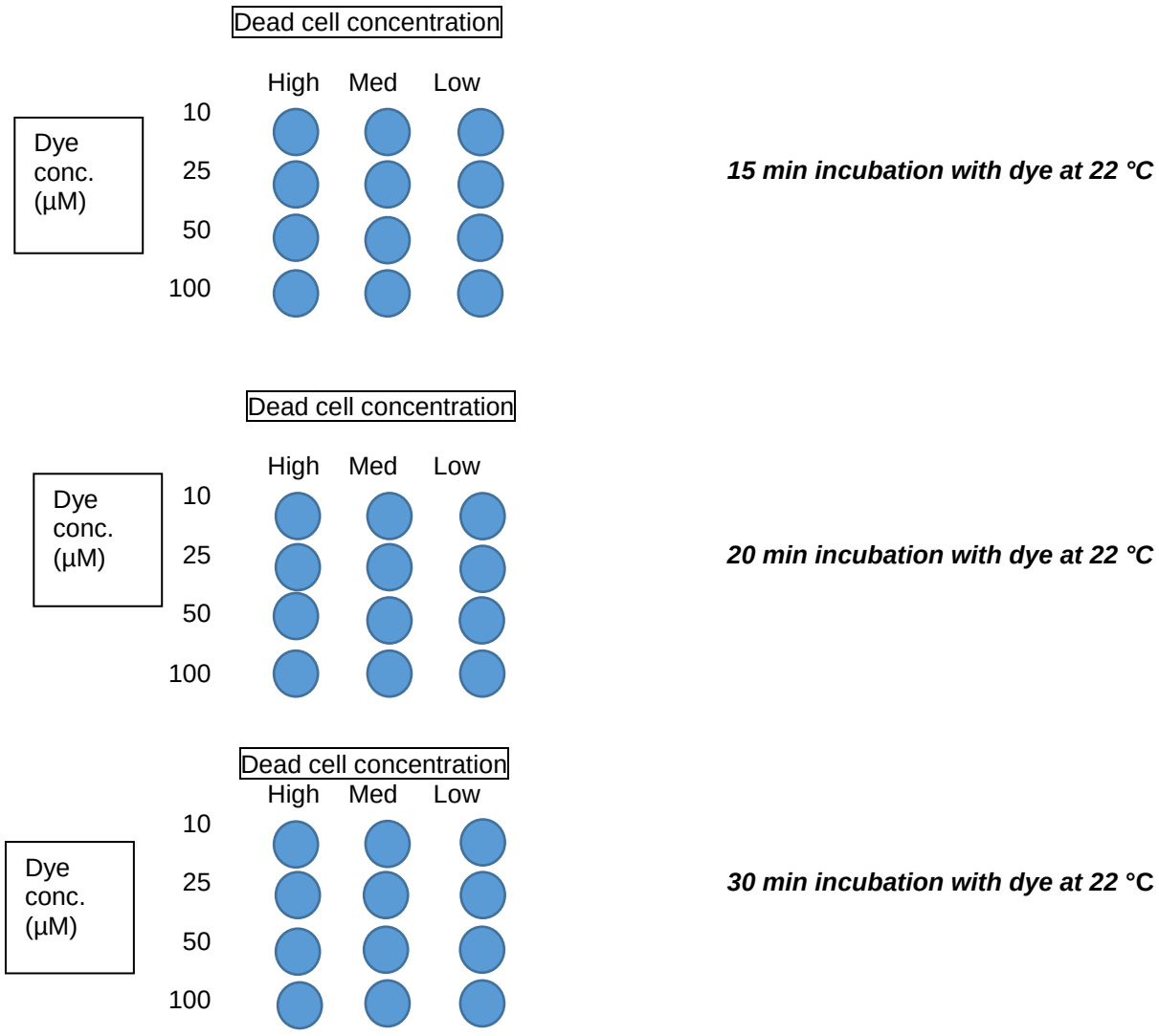

Figure 1. Simplified experimental design to optimise dye concentration and incubation time (one replicate for PEMAX, without controls).

The same set of controls were used for all experiments, however, dye specific controls were used (i.e., control with dye added).

3.7 INCUBATION TEMPERATURE

Yersinia ruckeri

The suitability of PEMAX and EMA dye treatment was assessed using mixtures of live and dead *Y. ruckeri* cells. All samples contained the same number of cells (10^9 CFU mL^{-1}) with adjustment made to the ratio of live to dead. Live cells (100, 80, 60, 40, 20 and 0 %) were mixed with dead cells and incubated at either: 0 (Ice), 4, 22or 30 °C.

Experiments were carried out using the previously optimised conditions and performed in triplicate (Figure 2).

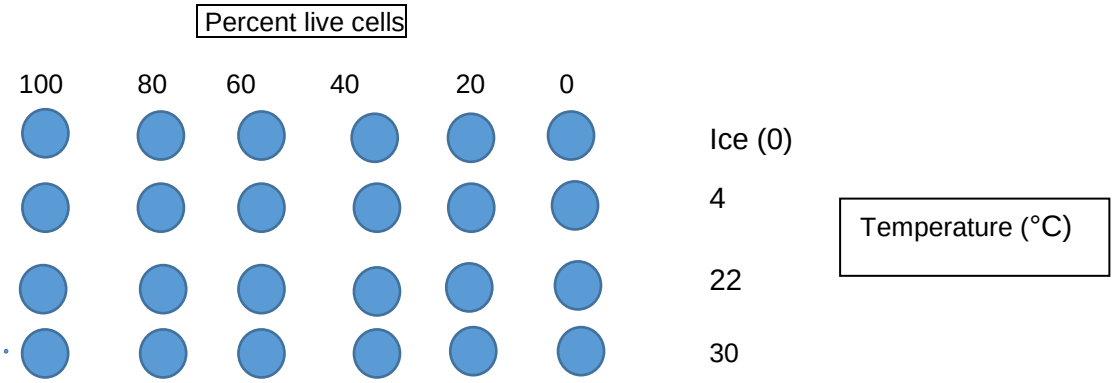

Figure 2. Simplified experimental design to optimise incubation time (one replicate of the *Y. ruckeri* experiment, without controls).

The ratios of % live cells for the *Y. ruckeri* experiment did not provide information additional to that gained from the 100 % live or dead cells. For the remaining pathogens, only live and dead cells were used at three different pathogen concentrations.

***Tenacibaculum maritimum* and ABV**

For *T. maritimum* concentrations of 10^8, 10^5 and 10^3 CFU mL^{-1} were used. The following concentrations were used for ABV: 10^7, 10^5 and 10^4 $TCID_{50}$. All cells were incubated at the same temperatures as *Y. ruckeri* using the previously optimised conditions (Section 3.6).

The following controls were used for all experiments (except where indicated):

- Dead cells with no treatment.
- Live/dead cells in ratios with no dye added and no exposure to light (*Y. ruckeri* only).
- Live cells with no treatment.

All controls were performed at RT incubation (~22 °C).

3.8 PHOTOPERIOD

Photoperiod optimisation was carried out on dead and live cells using the previously optimised conditions (Sections 3.6 – 3.7). Optimisation was performed using each dye with samples exposed to light for either: 5, 10 or 15 min.

The following bacterial concentrations were used: *Yersinia ruckeri*, 10^9 and 10^5 CFU mL^{-1}; *Tenacibaculum maritimum* 10^8, 10^5 and 10^3 CFU mL^{-1} (Figure 3). This experiment was not carried out with ABV as complete suppression of dead cells was observed during previous optimisation (Section 3.7).

The following experimental controls were used:

- Dead cells with no treatment at all bacterial concentrations.
- Live cells with no treatment at all bacterial concentrations.

The same set of controls were used for all experiments that were dye specific (i.e., control with dye added).

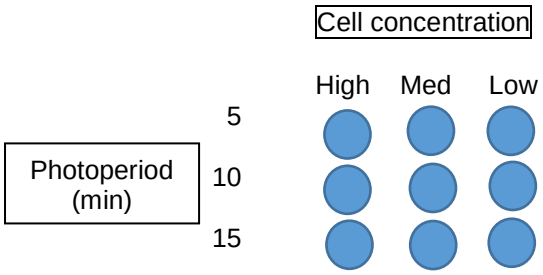

Figure 3. Simplified experimental design to optimise photoperiod (one replicate of the *T. maritimum* experiment, PEMAX dye, without controls).

3.9 RESUSPENSION BUFFERS

Only high bacterial concentrations of both live cells and dead cells were used for each resuspension buffer for *Yersinia ruckeri* and *Tenacibaculum maritimum* (10^9 and 10^8 CFU mL^{-1}, respectively). Pure cultures were used under the previously optimised conditions (Sections 3.6 – 3.8). Samples were processed using each dye. This experiment was not carried out with ABV as complete suppression observed during previous optimisation (Section 3.7).

Pellets of 1 mL of an overnight (*Y. ruckeri*) or 48 h culture (*T. maritimum*) were used with the pellets re-suspended in 245 µL of one of each of the following resuspension buffers:

- SD: 0.01, 0.03, 0.1 and 0.3 %.
- Triton X-100: 0.1, 0.5 and 1 %.
- Reaction buffer+ (1X).
- pH: 7, 7.5, 8 and 8.5.

- PBS (control).
- Nutrient broth, pH 7.2 (BHI and TYG-M for *Y. ruckeri* and *T. maritimum*, respectively).
- 0.85 % saline (*T. maritimum* only).
- Artificial seawater, 33 ppt (salt), pH 8.2 (*T. maritimum* only).

The following experimental controls were used:

- Dead cells with no treatment.
- Live cells with no treatment.

3.10 TROUBLESHOOTING MEASURES

3.10.1 Use of double dye exposure

To increase the differentiation between live and dead cells by vPCR, a double dye exposure was trialled on pure cultures of *Yersinia ruckeri* and *Tenacibaculum maritimum*.

Experiments were performed on high bacterial concentrations only (final concentration of: *Y. ruckeri* = 10^8 CFU $mL^{-1;}$ *T. maritimum* = 10^7 CFU mL^{-1}) with both EMA and PEMAX dye.

Yersinia ruckeri

Aliquots (500 µL) of an overnight culture of separate live and dead cells were added to vPCR tubes. Cells were pelleted at 10,000 g for 3 min, supernatant carefully removed and the pellet re-suspended in 0.1 % Triton X-100. Homogeneity of the solution ensured by repeat pipetting and vortexing. Dye was then added and the sample incubated. Three dye treatments were assessed for this experiment:

1) 100 µM of dye.
2) 50 µM of dye, double exposure (100 µM total).
3) 100 µM of dye, double exposure (200 µM total).

After incubation, the sample was pelleted as above, supernatant removed and the pellet re-suspended in PBS. Extra dye was added for treatments 2 and 3 only. All samples were incubated for 15 min and exposed to 15 min of light as per previous optimisation (Sections 3.6 and 3.8).

Tenacibaculum maritimum

Aliquots (500 µL) of a 48 h culture of separate live and dead cells were added to vPCR tubes. Cells were pelleted by centrifuging at 14,000 g for 5 min, the broth was removed and the pellet re-suspended in 0.85 % saline. Homogeneity of the solution was ensured by repeat pipetting and vortexing. Two dye treatments were assessed for this experiment:

1) 50 µM of dye.
2) 50 µM of dye, double exposure (100 µM total).

Dye was added and the sample incubated. After incubation, the sample was then pelleted as above, supernatant removed and the pellet re-suspended in PBS. Extra dye was added to treatment 2 only. All

samples were incubated for 15 min before being exposed to 15 min light as per previous optimisation (Sections 3.6 and 3.8).

The following experimental controls were used:

- Live, no treatment.
- Dead, no treatment.

3.10.2 Longer incubation time for *Yersinia ruckeri* with dye

This experiment was undertaken for *Y. ruckeri* to yield further differentiation between live and dead cells. Aliquots (500 µL; final concentration of 10^8 CFU mL^{-1}) were prepared as above with separate live and dead samples.

PEMAX was added to the samples (2 x 100 µM) and incubated for: 30, 45, 60, 75 or 90 min. Samples were then processed as per the optimised protocol (Sections 3.6 and 3.9).

The following experimental controls were used:

- Live, no treatment.
- Dead, no treatment.

3.10.3 "Double tube" method for *Yersinia ruckeri*

This experiment was undertaken for *Y. ruckeri* to try to further differentiate between live and dead cells and to reduce any amplification seen in the qPCR.

An overnight culture of *Y. ruckeri* was used and two concentrations of live and dead cells were carried out separately at final concentrations of 10^8 and 10^5 CFU mL^{-1}, respectively.

Three treatments were carried out for each cell concentration:

1. Single tube (vPCR tube) – control samples.
2. Double tube method (vPCR tubes).
3. Double tube method with dark tubes used for the first step to assess light sensitivity (amber tubes, vPCR tubes).

For treatment 1, the standard procedure was used on 3 x live and 3 x dead cultures with the optimal parameters (double dye exposure, 15 min incubation on ice with 15 min light exposure).

For treatment 2, the standard procedure was used on 3 x live and 3 x dead cultures with the optimal parameters (double dye exposure, 15 min incubation on ice with 15 min light exposure). Prior to dye exposure, the sample was transferred into a new vPCR tube, however both tubes were exposed to light.

For treatment 3, the standard procedure was used on 3 x live and 3 x dead cultures in amber tubes (light protective tubes) with the optimal parameters (double dye exposure, 15 min incubation on ice with 15

min light exposure). Prior to the dye exposure, the sample was transferred into a new vPCR tube with both the amber tube and the vPCR tube being exposed to light.

Extraction methods were applied to the first tube from the double tube methods (i.e., treatments 2 and 3) to determine any DNA debris left in the tube. The second tube from the double tube method contained the sample.

3.10.4 Washing prior to dye exposure

Pure cultures of live and dead *Y. ruckeri* (10^8 CFU mL^{-1}) were prepared as previously described.

Samples were treated one of three ways:

1. Washed once.
2. Washed twice.
3. Not washed.

Washing steps were carried out as follows: an aliquot (500 µL) of bacterial culture was pelleted by centrifugation at 10,000 g for 3 min. The supernatant was removed and the pellet re-suspended in 500 µL PBS and mixed until homogenous. This process was repeated for the washed twice treatment.

The following experimental controls were used:

- Live, no treatment.
- Dead, no treatment.

After washing, the samples were processed as per the optimised conditions for vPCR (Sections 3.6 – 3.9).

3.11 PARAMETERS FOR HIGH-THROUGHPUT PROCESSING

Transfer of the assay to a high-throughput platform (96-well plate in the photoactivation universal light, PAUL) was assessed for both *Tenacibaculum maritimum* and ABV. The vPCR tube and 96-well plate format were run in parallel.

Tenacibaculum maritimum

Five tissue homogenates were prepared as 1:10 dilutions in resuspension buffer (e.g., 100 mg tissue and 1 mL 0.85 % saline). From each of the tissue homogenates, aliquots (100 µL) were removed and placed into a vPCR tube and into a 96-well plate. To the remaining homogenates, bacteria were added (90 µL) and mixed to make the following final concentrations:

1) Dead, 10^4 CFU mL^{-1}.
2) Live/dead, 10^4 CFU mL^{-1}.
3) Live, 10^4 CFU mL^{-1}.

4) Dead, 10^6 CFU mL^{-1}.
5) Live/dead, 10^6 CFU mL^{-1}.

ABV

ABV at a neat concentration (final concentration 10^4 $TCID_{50}$) only was used due to the LOD of the assay and the degradation of RNA from dead virus following incubation in tissue. Three homogenates were prepared as a 1:10 dilution. From each of the tissue homogenates, aliquots (100 µL) were removed and placed into a vPCR tube and into a 96-well plate. To the remaining homogenates, virus (90 µL) was added as follows:

1) Dead, neat.
2) Live/dead, neat.
3) Live, neat.

For both pathogens, three aliquots (100 µL) of each sample were then added to vPCR tubes and the 96-well plate. The samples were then processed using the optimised protocol.

3.12 PROTOCOL FOR SPIKED TISSUE

Homogenised tissue was created for each tissue type (*Yersinia ruckeri* = kidney, *Tenacibaculum maritimum* = skin, ABV = liver) as a 1:10 dilution in the optimal resuspension buffer e.g., 100 mg of tissue in 1 mL of resuspension buffer (Section 3.9).

The suitability of dye treatment with mixed cells in tissue samples was assessed. All samples contained the same number of cells with adjustment made to the ratio of live to dead. Live cells (100, 80, 60, 40, 20 and 0 %) were mixed with dead cells. The following final cell concentrations were tested: *Y. ruckeri* = 10^8 and 10^4 CFU mL^{-1}; *T. maritimum* = 10^6 and 10^3 CFU mL^{-1}; ABV = 10^4 $TCID_{50}$ and 10^3 $TCID_{50}$.

The following experimental control were used for each tissue:

- Tissue homogenate not spiked and not treated.

3.13 PROTOCOL FOR REPEATABILITY ON ARTIFICIALLY SPIKED TISSUE

Fish tissue homogenates were created using a 1:10 dilution of tissue to resuspension buffer as per Section 3.12. For each pathogen, aliquots of homogenates (100 µL) were spiked with ratios of live and dead pathogens (100 µL) at two different final concentrations.

Yersinia ruckeri

- 10^3 CFU mL^{-1}:
    - 100 % dead.
    - 50 % live.
    - 100 % live.
- 10^7 CFU mL^{-1}:
    - 100 % dead.
    - 50 % live.

Tenacibaculum maritimum

- 10^3 CFU mL^{-1}:
    - 100 % dead.
    - 50 % live.
    - 100 % live.
- 10^5 CFU mL^{-1}:
    - 100 % dead.
    - 50 % live.

ABV

- 10^4 $TCID_{50}$:
    - 100 % dead.
    - 50 % live.
    - 100 % live.
- 10^3 $TCID_{50}$:
    - 100 % dead.
    - 50 % live.

The experiment was carried out three times and each time in triplicate, e.g., nine repeats for each sample.

The following experimental controls were used for each tissue:

- For each sample, one homogenate that was spiked with no treatment (n = 3 per sample).
- For each experiment, one homogenate not spiked and with no dye treatment (n = 3).

3.14 PROFICIENCY TESTING

Yersinia ruckeri

Proficiency testing was carried out both internally (2 users at AHL) and externally (1 user at PHEL). A panel of 20 spiked tissue samples was prepared and tested as per instructions (Appendix 1). Each sample was divided into two of which one was treated with PEMAX. A neat suspension of the bacterial culture was extracted in parallel and run as a dilution series in the qPCR.

Tenacibaculum maritimum

Proficiency testing was only carried out internally (2 users at AHL) due to the bacteria being held in the PC3+ laboratory. A panel of 20 spiked tissue samples was prepared and tested with 2 users in parallel (Appendix 2). Each sample was divided into two of which one was treated with PEMAX. A neat suspension of the bacterial culture was extracted in parallel and run as a dilution series in the qPCR.

ABV

Proficiency testing was only carried out internally (2 users at AHL) due to the virus being held in the PC3+ laboratory. A panel of 20 spiked tissue samples was prepared and tested by 2 users in parallel. Each sample was divided into two of which one was treated with PEMAX (Appendix 3). A neat suspension of the virus was extracted in parallel and run as a dilution series in the conventional RT-PCR.

3.15 DETERMINING A CUT-OFF VALUE FOR QPCR

As complete suppression was not observed in the v-qPCR, a cut-off value was required to be able to determine the presence of live cells in a sample. The following steps were taken to determine the % difference of the change (Δ) in Ct value between samples that were not treated (-PEMAX) and treated (+PEMAX):

- Δ Ct = Mean Ct^{+PEMAX} – Mean Ct^{-PEMAX}.
- % Δ Ct^{+PEMAX} = Difference/Mean Ct^{+PEMAX}.
- % Δ Ct^{-PEMAX} = Difference/Mean Ct^{-PEMAX}.
- % Δ Ct = % Δ Ct^{-PEMAX} - % Δ Ct^{+PEMAX}.

All results from experiments using the optimised protocol were compiled and the % difference value determined (*Yersinia ruckeri n* = 85, *Tenacibaculum maritimum n* = 103). A conservative % was ascribed based on samples that contain dead, live or a mixture of cells.

3.16 PROTOCOL FOR NATURALLY INFECTED FISH TISSUES WITH GRAM-NEGATIVE BACTERIA

Yersinia ruckeri

Kidney samples from fish naturally infected with *Y. ruckeri* from a freshwater salmon farm were sourced from both fresh (*n* = 12) and frozen (*n* = 20) material. Kidney tissue (100 mg) was homogenised with 1 mL 0.1 % Triton X-100. Aliquots of the homogenate (100 µL) were then processed in a 96-well plate and subjected to one of the following treatments:

1) Heat treated + PEMAX.
2) + PEMAX.
3) – PEMAX.

The optimised protocol was then performed (Sections 3.6 – 3.10). Each homogenate (100 µL) was also plated onto BA to assess cell viability. Agar plates were incubated at 22 °C for 7 d.

Tenacibaculum maritimum

Skin lesion samples (*n* = 30) from fish suspected to be naturally infected with *T. maritimum* were sourced from a marine salmon farm. Samples arrived at the AHL on ice the day after collection. Skin lesion tissue (100 mg) was homogenised with 1 mL 0.85 % saline. Aliquots of the homogenate (100 µL) were then processed in a 96-well plate and subjected to one of the following treatments:

1) Heat treated + PEMAX.
2) + PEMAX.
3) – PEMAX.

The optimised protocol was performed and the qPCR and conventional PCR run on all samples in duplicate. Each homogenate (100 µL) was also plated onto Anacker & Ordal + sea salt (an alternative supportive media for *T. maritimum* to TYG-M) to assess cell viability. Each sample was tested at neat, 10^{-1} and 10^{-2} dilutions. Agar plates were incubated at 22 °C for 14 d.

A second batch of skin lesion samples (*n* = 32) from fish suspected to be naturally infected with *T. maritimum* was sourced from the same location as the first batch. Chilled samples arrived at the AHL less than 48 hours after collection. Samples were processed as above as well as being processed using the enrichment protocol (Section 3.17).

ABV

Naturally infected tissue was unable to be sourced for this project.

3.17 ENRICHMENT PROTOCOL PRIOR TO DYE TREATMENT

This experiment was undertaken for *Tenacibaculum maritimum* to achieve more consistent results in samples that contained low cell concentrations (≤ 10^4 CFU mL^{-1}). The following final concentrations of *T. maritimum* were assessed in triplicate:

- 10^4 CFU mL^{-1} live/10^6 CFU mL^{-1} dead.
- 10^3 CFU mL^{-1} live/10^6 CFU mL^{-1} dead.
- 10^2 CFU mL^{-1} live/10^6 CFU mL^{-1} dead.
- 10^1 CFU mL^{-1} live/10^6 CFU mL^{-1} dead.
- 10^5 CFU mL^{-1} live/10^3 CFU mL^{-1} dead.
- 10^4 CFU mL^{-1} live.
- 10^5 CFU mL^{-1} live.
- 10^3 CFU mL^{-1} dead.
- 10^4 CFU mL^{-1} dead.

Samples were homogenised as per Section 3.16 and treated in two ways:

- Processed for vPCR immediately.
- Enriched prior to vPCR processing.

Immediately processed samples were divided into two aliquots (100 µL) one of which was treated with PEMAX. Enrichment was carried out by incubating 300 µL of the homogenate in 3 mL of TYG-M broth and incubating at 22 °C for 48 h. The broth culture (2 mL) was then pelleted by centrifugation at 14,000 g for 5 min. The supernatant was removed and the pellet was re-suspended in 200 µL of saline. The sample was then divided into two (2 x 100 µL) of which one sample was treated with PEMAX. Samples were then processed using the optimal vPCR protocol in 96-well plate format (Section 3.11).

This method was also trialled on 30 naturally infected tissue samples that had been stored at -20 °C and seven fresh samples.

3.18 STATISTICAL ANALYSIS

Statistical analysis was performed in R studio, version 0.98.501 (R Core Team 2015).

A generalised linear model (GLM) was performed. For tests where a qPCR was used, the response variable was the Ct value. These were log transformed to meet the assumption of normality (family = Gaussian). For conventional PCR, the response variable was the binomial result (family = binomial).

The specific pair-wise differences between groups was tested using Tukey contrasts and *p*-values were adjusted using the Benjamini & Hochberg method (R package multcomp, Hothorn et al., 2008).

The significance of the explanatory variables in all models was assessed using likelihood ratio tests (Zuur et al., 2009). *p*-values < 0.05 were considered statistically significant.

The effect of the dye concentrations and incubation times on assay outputs were assessed using a comparison of the Ct value between treatment groups. To assess the effect of incubation temperatures and photoperiods on assay outputs, the Ct values of dead samples were compared with each other. .Dead samples were also compared with live samples as well as with the control groups.

The effect of resuspension buffers, double dye exposure, washing and longer incubation on assay outputs was assessed by comparing Ct values from live and dead samples separately, as well as comparing the Ct values from live, dead and control samples.

A correlation co-efficient was carried out in Microsoft Excel to determine the relationship between the varying concentrations of live cells in a tissue matrix (section 4.14).

For the high-throughput experiment, the Ct value of samples were compared between the standard and high-throughput assays. Ct values between the different users were compared for proficiency testing.

Statistical outputs are provided in Appendix 5.

4 Results

4.1 ASSAY SENSITIVITY

Yersinia ruckeri

The LOD for *Y. ruckeri* was determined to be 10^4 CFU mL^{-1}. The Ct value did not increase at lower bacterial concentrations.

The efficiency of the v-qPCR (10^2 to 10^7 CFU mL^{-1}) was 318 % and the efficiency of the qPCR at the same bacterial concentration range was 100 % (Figure 4). The amplification efficiency should ideally be close to 90 % with 100 % being the most efficient. The high % efficiency in the v-qPCR indicates that the dead cells or the dye are impacting the efficiency of the assay, however the actual number of bacterial cells detected was the same between qPCR and v-qPCR. Amplification efficiencies of the v-qPCR in the range of the LOD was 151 % with the qPCR being 93 % (Figure 5). This indicates that the results from the v-qPCR will not be reliable at Ct values > 32.

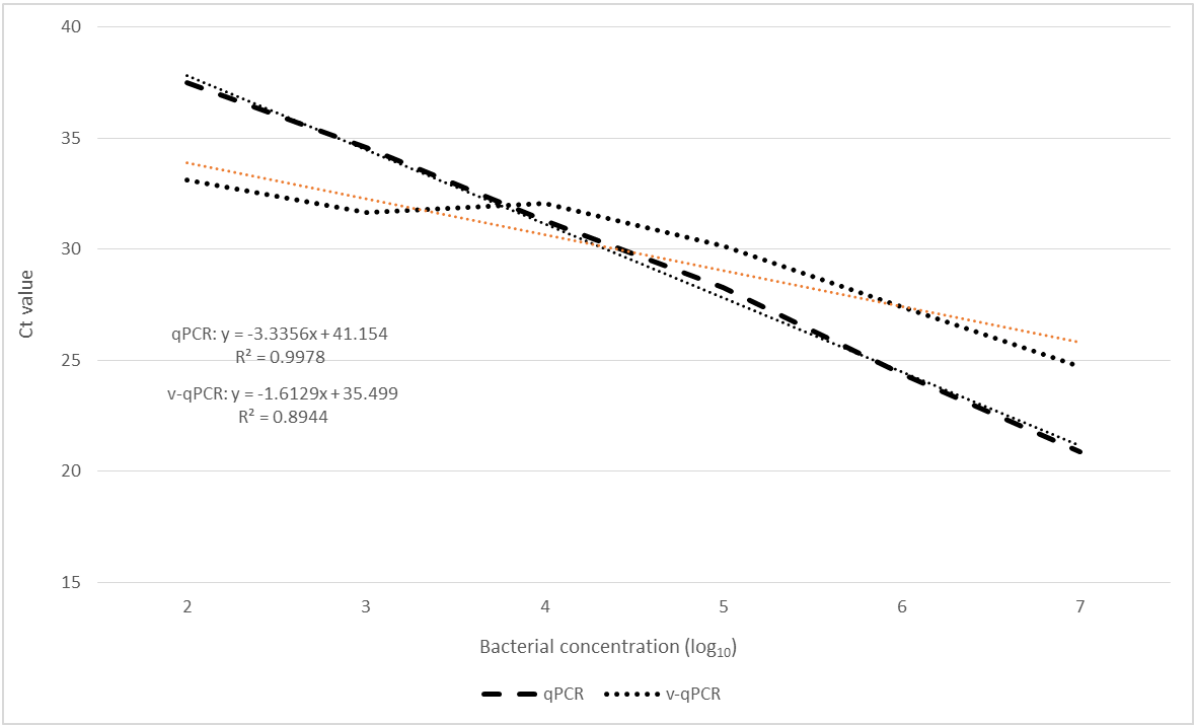

Figure 4. Amplification efficiencies of q-vPCR and vPCR over a dilution series of live *Y. ruckeri* cells.

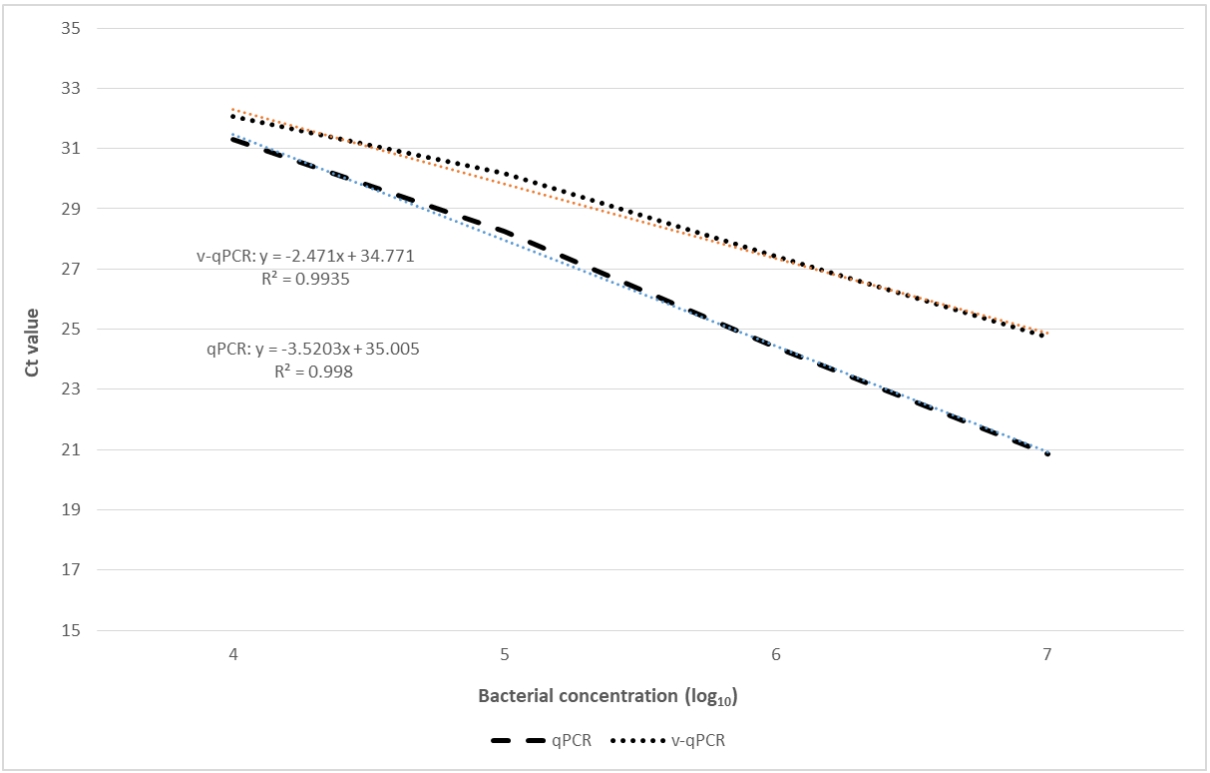

Figure 5. Amplification efficiencies of q-vPCR and vPCR over a dilution series of live *Y. ruckeri* cells in the range of the LOD (> 10^4 CFU mL^{-1}).

Bacteria that were not treated with dye maintained a similar Ct value for all concentrations (Figure 6).

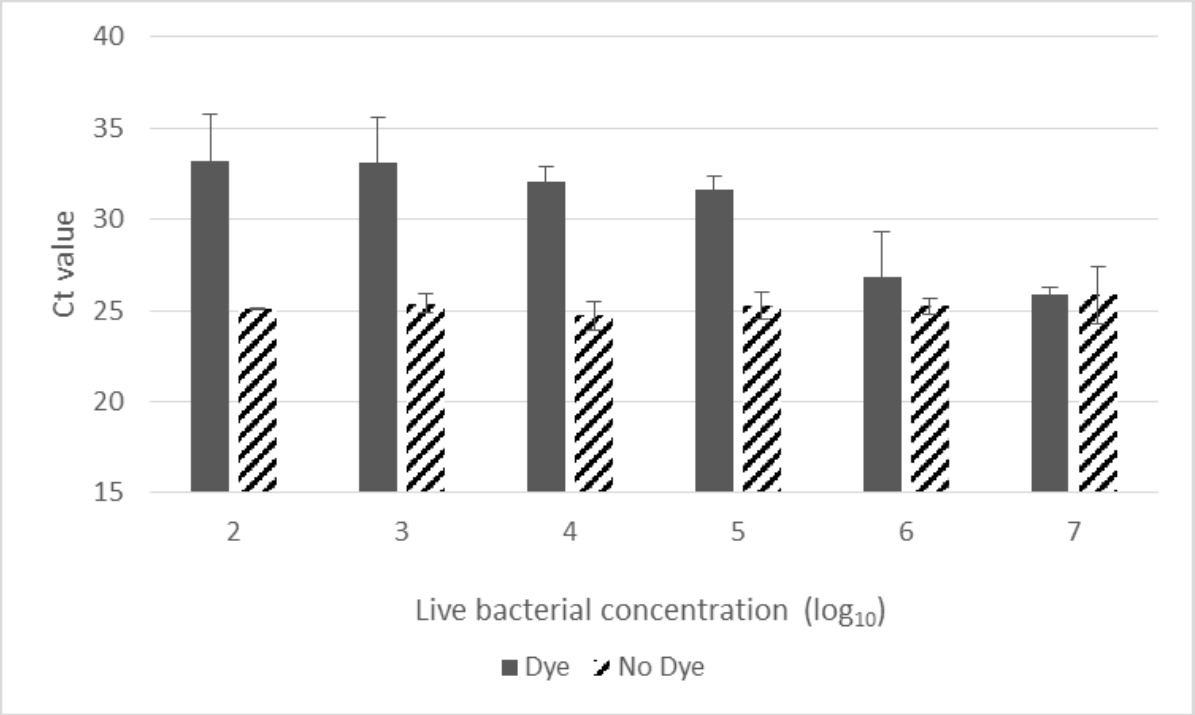

Figure 6. Bars represent average Ct values from the qPCR for *Yersinia ruckeri* dilution series of live cells in a background of a high concentration of dead cells (10^8 CFU mL^{-1}) and either treated with dye (block colour bar), or not treated with dye (lined bar). *n* = 3. Error bars represent the standard deviation (SD) between replicates.

Tenacibaculum maritimum

The LOD for *T. maritimum* was determined to be 10^3 CFU mL^{-1}. The Ct value did not increase below this bacterial concentration (Figure 7).

The amplification efficiency of the v-qPCR assay from 10^1 – 10^6 CFU mL^{-1} was 223 % and the qPCR amplification efficiency was 134 %. When assessing the efficiency within the range of the LOD range (> 10^3 CFU mL^{-1}) the v-qPCR efficiency was 86 % and qPCR was 105 % (Figure 8). The amount of bacterial cells detected between the qPCR and the v-qPCR was the same, but the v-qPCR had a reduced efficiency. These results indicate that v-qPCR results will not be reliable at Ct values of > 36.

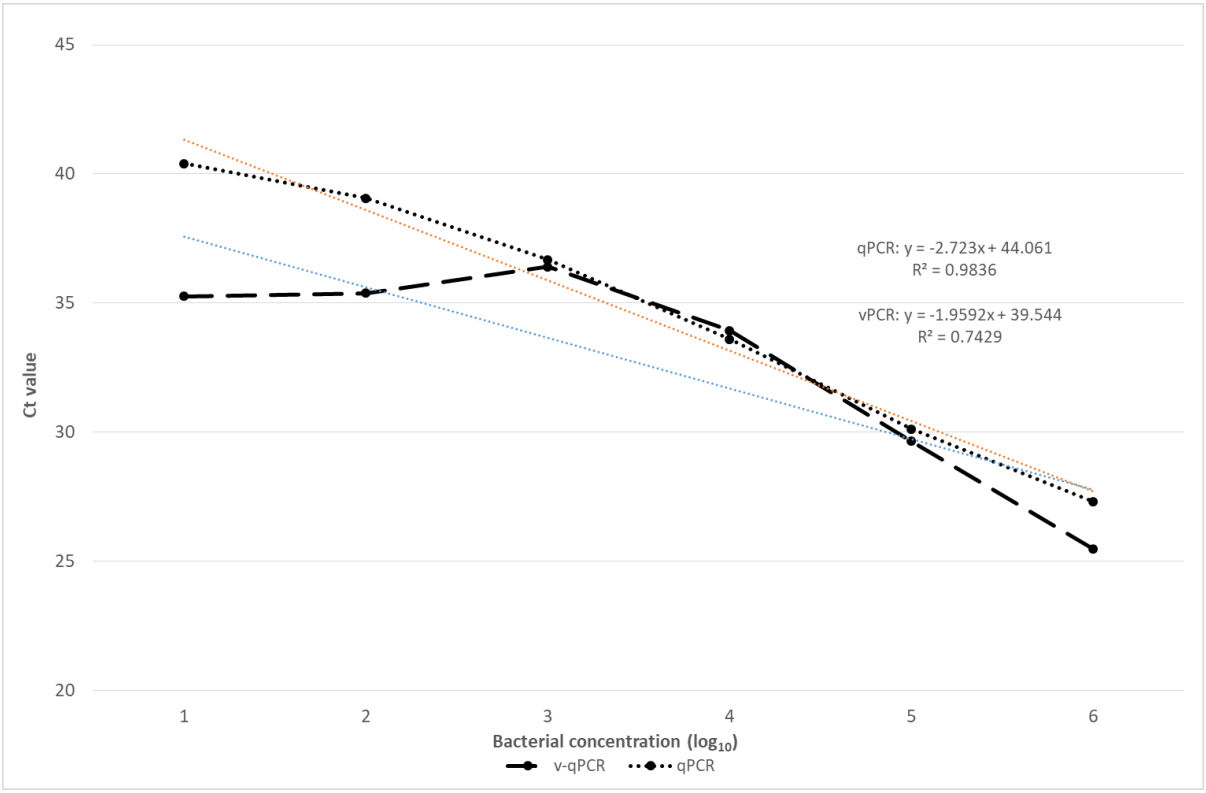

Figure 7. Amplification efficiencies of *T. maritimum* q-vPCR and vPCR over a dilution series of live bacterial cells.

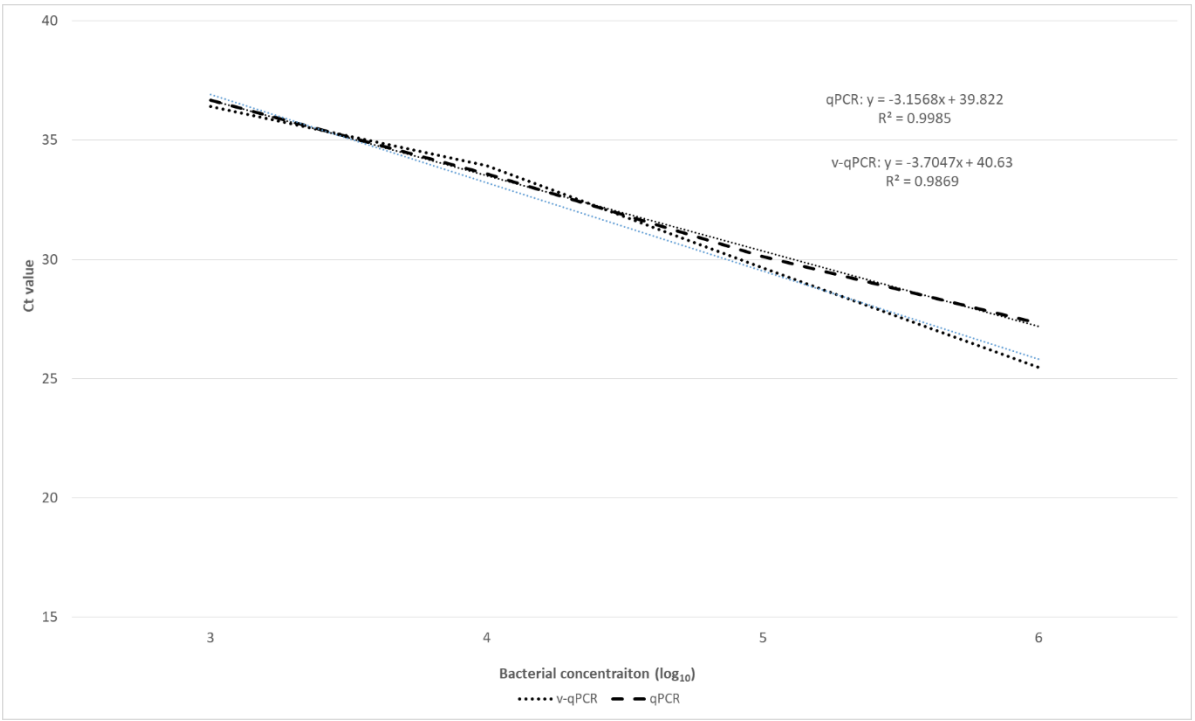

Figure 8. Amplification efficiencies of the *T. maritimum* q-vPCR and vPCR over a dilution series within the LOD (> 10^3 CFU mL^{-1}).

Bacteria that were not treated with dye maintained a similar Ct value for all concentrations (Figure 9).

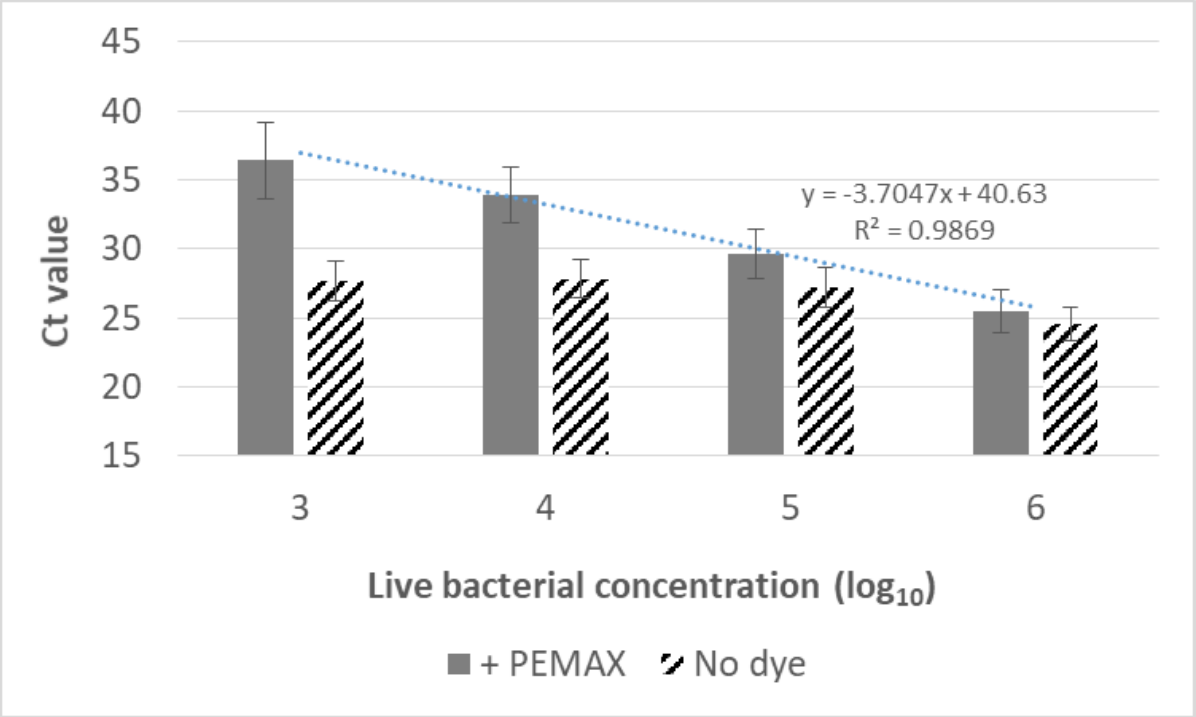

Figure 9. Bars represent average Ct values from the qPCR for *T. maritimum* dilution series of live cells in a background of a high concentration of dead cells (10^6 CFU mL^{-1}) and either treated with dye (block colour bar), or not treated with dye (lined bar). *n* = 3. Error bars represent the SD between replicates.

The LOD for the nested conventional PCR for *T. maritimum* in both dye treated and not dye treated live cells was 10^3 CFU mL^{-1}. Below this bacterial concentration, detection of amplicons was not consistent.

ABV

The LOD for live virus treated with and without dye were identical at 10^4 $TCID_{50}$. Amplicons were repeatedly produced in the RT-PCR at this concentration.

4.2 HEAT KILL TREATMENT

An overnight culture of *Yersinia ruckeri* yielded 2.8 x 10^9 CFU mL^{-1} and a 48 h culture of *Tenacibaculum maritimum* yielded 1 x 10^8 CFU mL^{-1}. Heat treatment (99 °C for 10 min) of both of these bacterial pathogens achieved a complete kill. This regime was used for all heat treated samples in the subsequent experiments.

Growth of ABV for 7 days yielded 2.7 x 10^7 $TCID_{50}$. In contrast to the bacteria, heating at 99 °C for 20 min achieved a complete kill and was used for all heat treated samples in the subsequent experiments.

4.3 OPTIMISING PEMAX AND EMA DYE CONCENTRATION

Yersinia ruckeri

Bacterial concentrations of 10^2 CFU mL^{-1} did not give consistent results as this was below the LOD of the assay. These results were not used for statistical analysis for EMA. This experiment was repeated using 10^9 and 10^7 to 10^3 CFU mL^{-1} with PEMAX dye only.

The high PEMAX concentration of 100 µM was significantly more effective (i.e., resulted in higher Ct values) than all lower dye concentrations at bacterial concentrations of 10^9 to 10^7 CFU mL^{-1} (Figure 10). No significant difference was observed between the dye concentrations applied to lower bacterial concentrations of 10^6 to 10^3 CFU mL^{-1}.

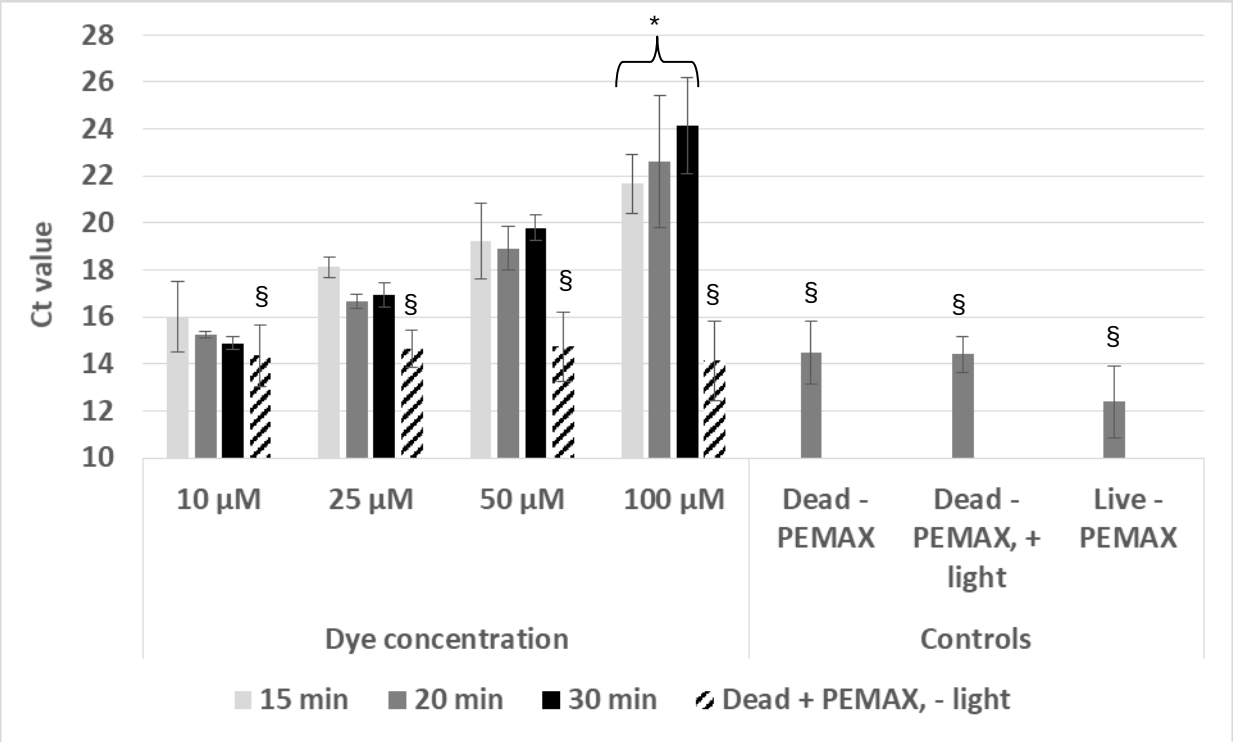

Figure 10. Bars represent average Ct value of dead *Y. ruckeri* (10^9 CFU mL^{-1}) exposed to varying concentrations of PEMAX dye with varying incubation times. $n = 3$. * = significant difference from 10, 25 and 50 µM ($p < 0.05$), § = significant difference from dead treated samples at all dye concentrations and incubation times ($p < 0.05$). Error bars represent the SD between the replicates.

The high EMA concentration of 100 µM were significantly more effective than the lower dye concentrations for 10^9 CFU mL^{-1} of *Y. ruckeri*. No significant difference was observed between the dye concentrations applied to lower bacterial concentrations (Figure 11).

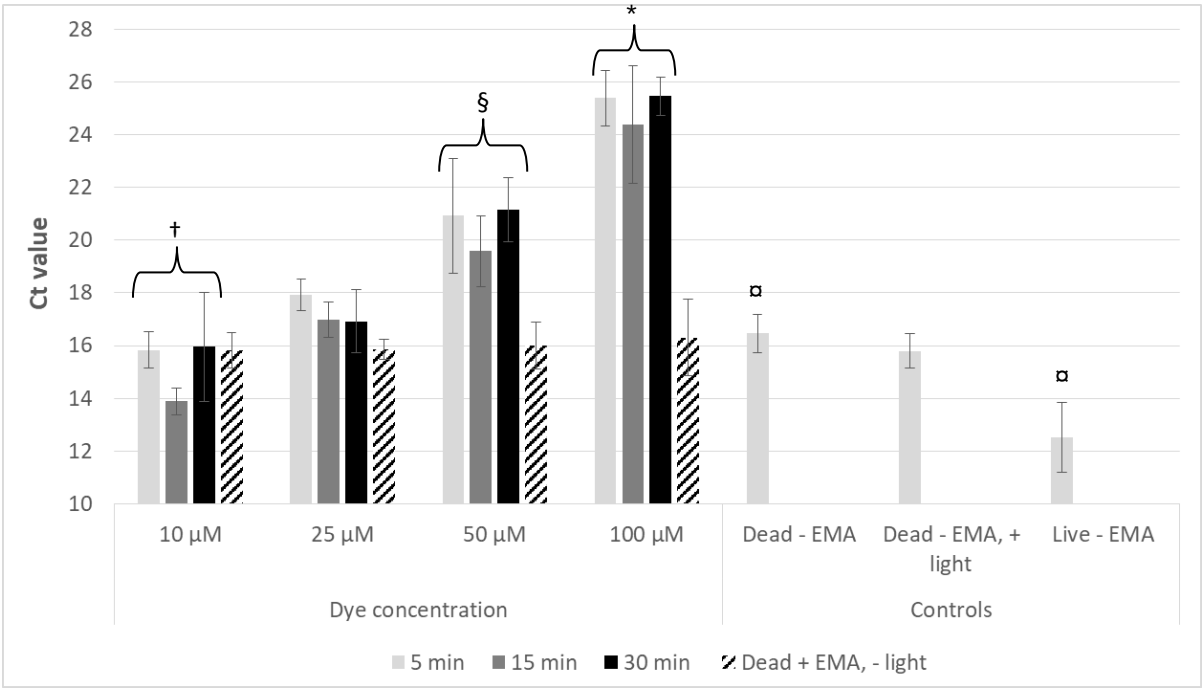

Figure 11. Bars represent average Ct value of dead *Y. ruckeri* (10^9 CFU mL^{-1}) exposed to varying concentrations of EMA and varying incubation times. *n* = 3. * = significant difference from 10, 25 and 50 µM ($p < 0.01$), † = significant difference from 25 and 50 µM ($p < 0.01$), § = significant difference from 25 µM ($p < 0.01$), ¤ = significant difference from dead treated samples at all dye concentrations and incubation times ($p < 0.01$). Error bars represent the SD between the replicates.

Conventional PCR was carried out on dead bacterial concentrations of 10^9, 10^5 and 10^2 CFU mL^{-1} for PEMAX and EMA treated samples. Complete suppression was not consistently observed in any bacterial concentration at any dye concentration or incubation time (Tables 1 and 2). The amplicons were visually less intense (intensity was not empirically measured) following the use of higher dye concentrations and longer incubation times, indicating these conditions were more effective at differentiating between live and dead cells.

Table 1: Conventional vPCR results for different PEMAX concentrations and incubation times assessed on dead *Y. ruckeri* cells. Results in the incubation time columns indicate the number of replicates out of three that produced an amplicon.

		Incubation time (min)			
Dye concentration (µM)	**Bacterial concentration (CFU mL^{-1})**	**15**	**20**	**30**	**Controls**
10	10^9	3	3	3	3
25	10^5	2	2	2	3
	10^2	0	1	2	2
	10^9	3	3	2	3
50	10^5	2	1	3	3
	10^2	0	0	2	3
	10^9	3	3	3	3
100	10^5	2	3	3	3
	10^2	2	1	2	1
	10^9	3	3	3	3
	10^5	2	2	2	3
	10^2	2	1	1	3

Table 2: Conventional vPCR results for different EMA concentrations and incubation times assessed on dead *Y. ruckeri* cells. Results in the incubation time columns indicate the number of replicates out of three that produced an amplicon.

		Incubation time (min)			
Dye concentration (µM)	**Bacterial concentration (CFU mL^{-1})**	**5**	**15**	**30**	**Controls**
10	10^9	3	3	3	3
	10^5	2	2	3	3
	10^2	2	2	2	3
25	10^9	3	3	3	3
	10^5	3	3	1	2
	10^2	3	3	3	3
50	10^9	3	3	3	3
	10^5	3	3	3	3
	10^2	3	3	2	3
100	10^9	3	3	3	3
	10^5	3	3	2	3
	10^2	3	3	1	3

A higher dye concentration was the most effective for both dyes. This is likely due to the differences in "free" nucleic acid available. When a high concentration of dead cells are present, an excess of dye is required to bind with any additional nucleic acid that are not enclosed in the cell.

Summary: Both PEMAX and EMA at 100 µM were used in subsequent *Y. ruckeri* experiments.

Tenacibaculum maritimum

Higher PEMAX dye concentrations (i.e., 50 and 100 µM) used on dead *T. maritimum* cells were more effective than lower concentrations (i.e., 10 or 25 µM). The higher concentrations were not significantly different from each other (Figure 12). As with *Y. ruckeri*, no significant differences were observed between the dye concentrations for lower bacterial concentrations.

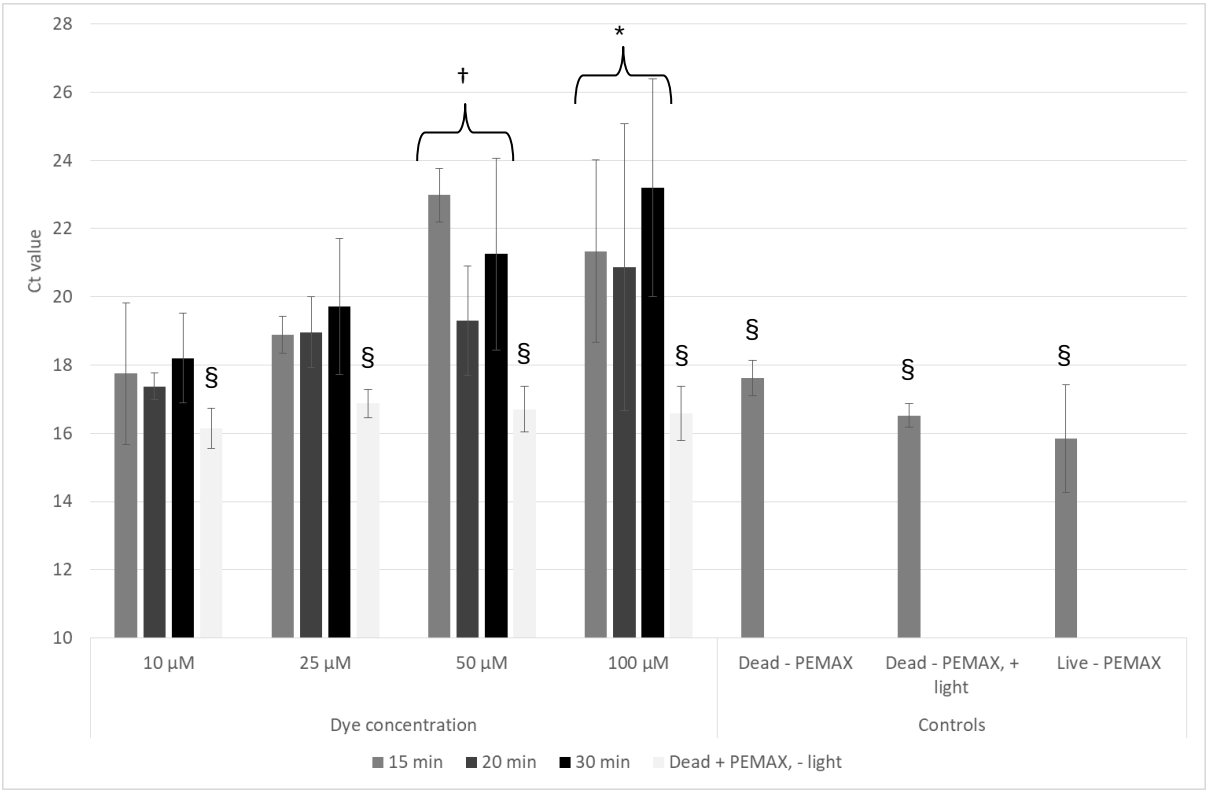

Figure 12. Bars represent average Ct value of dead *T. maritimum* (10^8 CFU mL^{-1}) exposed to varying concentrations of PEMAX and varying incubation times. *n* = 3. * = significant difference from 10 and 25 µM ($p < 0.05$), † = significant difference from 10 µM ($p < 0.01$), § = significant difference from dead treated samples at all dye concentrations and incubation times ($p < 0.05$). Error bars represent SD between replicates.

The use of different EMA concentrations used on dead *T. maritimum* cells were not significantly different for any of the bacterial concentrations used in this study (Figure 13).

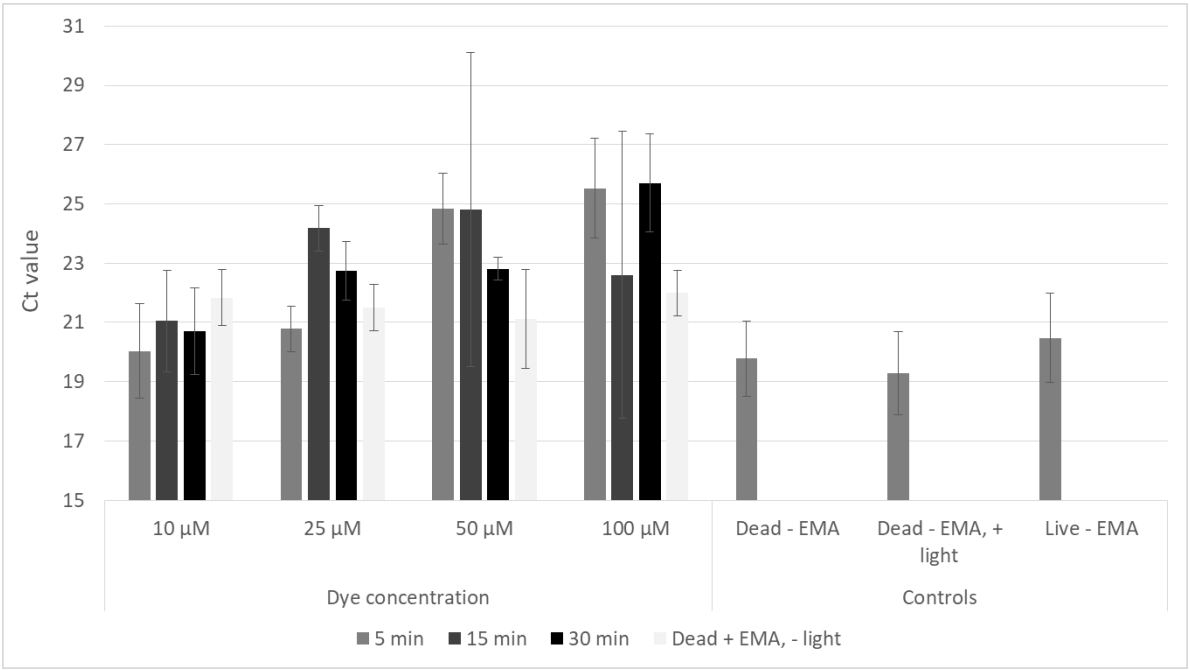

Figure 13. Bars represent average Ct value of dead *T. maritimum* (10^8 CFU mL^{-1}) exposed to varying concentrations of EMA and varying incubation times. *n* = 3. Error bars represent the SD between replicates.

For conventional vPCR, complete suppression was common at cell concentrations of 10^5 and 10^3 CFU mL^{-1} with both dyes at all concentrations (Tables 3 and 4). Only one replicate of 10^8 CFU mL^{-1} (100 µM

for 30 min incubation) was ever fully supressed with PEMAX. As this result was not consistent, 100 µM was not considered to be better than 50 µM.

Table 3: Conventional vPCR results for different PEMAX concentrations and incubation times assessed on dead *T. maritimum* cells. Results in the incubation time columns indicate the number of replicates out of three that produced an amplicon.

		Incubation time (min)			
Dye concentration (µM)	**Bacterial concentration (CFU mL^{-1})**	**15**	**20**	**30**	**Controls**
10	10^8	3	3	3	3
	10^5	0	2	2	3
	10^3	0	2	1	3
25	10^8	3	3	3	3
	10^5	2	2	0	3
	10^3	0	0	0	3
50	10^8	3	3	3	3
	10^5	3	2	2	3
	10^3	0	0	1	3
100	10^8	3	3	2	3
	10^5	1	1	2	3
	10^3	0	0	1	3

Table 4: Conventional vPCR results for different EMA concentrations and incubation times assessed on dead *T. maritimum* cells. Results in the incubation time columns indicate the number of replicates out of three that produced an amplicon.

		Incubation time (min)			
Dye concentration (µM)	**Bacterial concentration (CFU mL^{-1})**	**15**	**20**	**30**	**Controls**
10	10^8	3	3	3	3
	10^5	2	3	3	3
	10^3	2	2	2	3
25	10^8	3	3	3	3
	10^5	3	3	3	3
	10^3	3	3	3	3
50	10^8	3	3	3	3
	10^5	3	3	3	3
	10^3	3	3	2	3
100	10^8	3	3	3	3
	10^5	3	3	2	3
	10^3	3	3	2	3

Summary: A dye concentration of 50 µM was used for both PEMAX and EMA dyes in subsequent *T. maritimum* experiments.

ABV

No statistically significant difference was observed in any of the dye concentrations trialled on dead ABV cells.

Visual assessment of the electrophoresis gels showed that 100 µM PEMAX was the only concentration where amplification was completely supressed in all replicates of 10^7 $TCID_{50}$ dead virus (Figure 14). No

amplification was observed at any dye concentration applied to the lower viral dilutions (10^5 and 10^4 $TCID_{50}$).

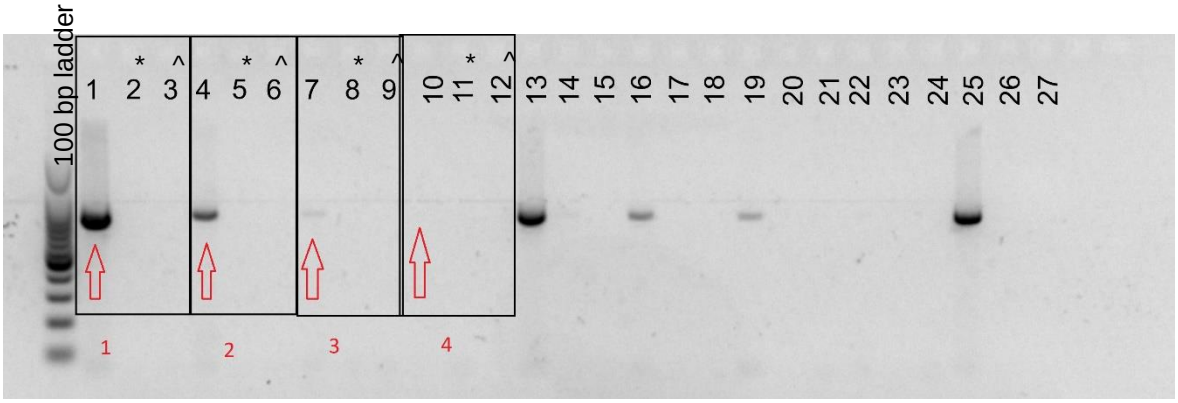

Figure 14. Gel electrophoresis image of dead ABV treated with PEMAX at different dye concentrations and incubation times. Box 1 = 10 µM, Box 2 = 25 µM, Box 3 = 50 µM, Box 4 = 100 µM. Arrows indicate the neat viral dilution. Box 1-4 were all incubated at 15 min. * = 10^5 $TCID_{50}$, ^ = 10^4 $TCID_{50}$.

No statistically significant differences were observed following application of any EMA concentration. The PCR product appeared to be less intense at the high viral concentration (10^7 $TCID_{50}$) when a high dye concentration (100 µM) was applied. However, full suppression of the PCR product was never seen (Figure 15). No amplification of dead virus was observed at concentrations of 10^5 and 10^4 $TCID_{50}$ treated with any dye concentrations.

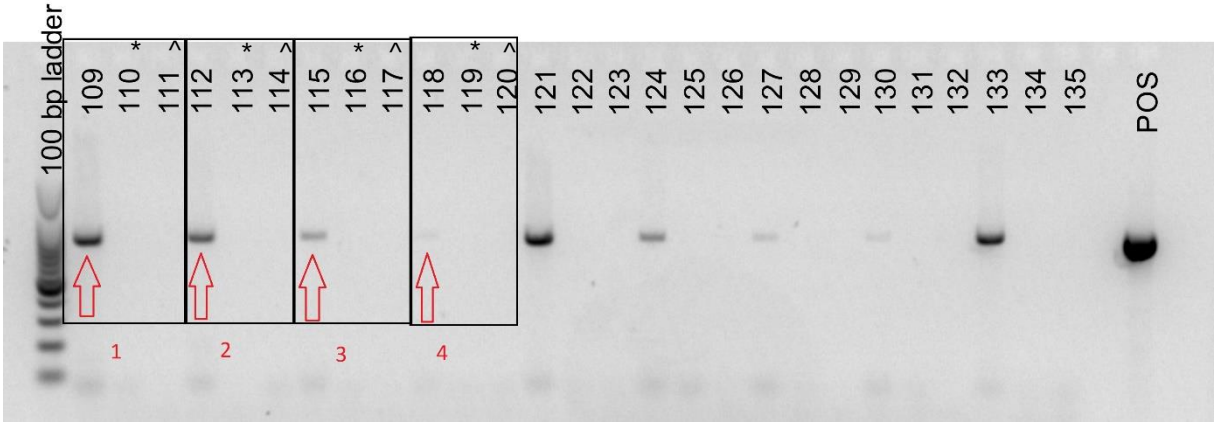

Figure 15. Gel electrophoresis image of dead ABV treated with EMA at different dye concentrations and incubation times. Box 1 = 10 µM, Box 2 = 25 µM, Box 3 = 50 µM, Box 4 = 100 µM. Arrows indicate the neat viral dilution. Box 1-4 were all incubated at 15 min. * = 10^5 $TCID_{50}$, ^ = 10^4 $TCID_{50}$.

All controls (live and dead virus) amplified concentrations of 10^7 and 10^5 $TCID_{50}$. Amplification was variable at the lowest dilution, 10^4 $TCID_{50}$ (live cells 1/3 amplified, dead cells 3/3 amplified).

Summary: A concentration of 100 µM was used for both PEMAX and EMA in subsequent ABV experiments.

4.4 OPTIMISING INCUBATION TIME

Yersinia ruckeri

For PEMAX v-qPCR, incubation time had no influence at the higher bacterial concentrations (10^9 to 10^5 CFU mL^{-1}). For the lower bacterial concentrations, incubation times above 20 min were optimal.

However, taking into account the results from the higher bacterial concentration, which appeared to be more consistent, a 15 min incubation time was chosen for subsequent experiments. Conventional vPCR did not consistently result in full suppression for any incubation time (Table 1).

For EMA v-qPCR, no significant differences were seen between any incubation times at any bacterial concentration. This result may suggest that EMA was penetrating the damaged cell walls of the cells in as little as 5 min. A 5 min incubation time was not assessed for PEMAX. Conventional vPCR did not show full suppression for any incubation time assessed (Table 2).

Summary: For PEMAX and EMA a 15 min incubation time used for subsequent *Y. ruckeri* experiments. Incubation time did not improve the effectiveness nor did this period have a negative impact on the results. In addition, this time period is more conducive to assay workflow.

Tenacibaculum maritimum

For PEMAX or EMA v-qPCR, no significant differences were observed between any incubation times evaluated at any of the bacterial concentrations assessed.

Conventional vPCR did not show full suppression for any incubation time assessed for either PEMAX or EMA treatment.

Summary: As no significant differences were seen between incubation times evaluated, a 15 min incubation time was used for both dyes in subsequent *T. maritimum* experiments.

ABV

For PEMAX or EMA conventional vRT-PCR, there were no significant differences observed between any incubation times or viral concentrations. The observed intensity of the amplicons on gel electrophoresis did not appear to be different between 15 and 30 min incubation.

Summary: As no significant differences were seen between incubation times evaluated, a 15 min incubation was used for both dyes in subsequent ABV experiments.

4.5 OPTIMISING INCUBATION TEMPERATURE

Yersinia ruckeri

For PEMAX v-qPCR used on high bacterial concentrations (i.e., 10^9 CFU mL^{-1}), a significantly greater Ct value was observed following incubation on ice (Figure 16). No significant differences were observed between any of the incubation temperatures assessed for cells at 10^5 CFU mL^{-1} (Figure 17).

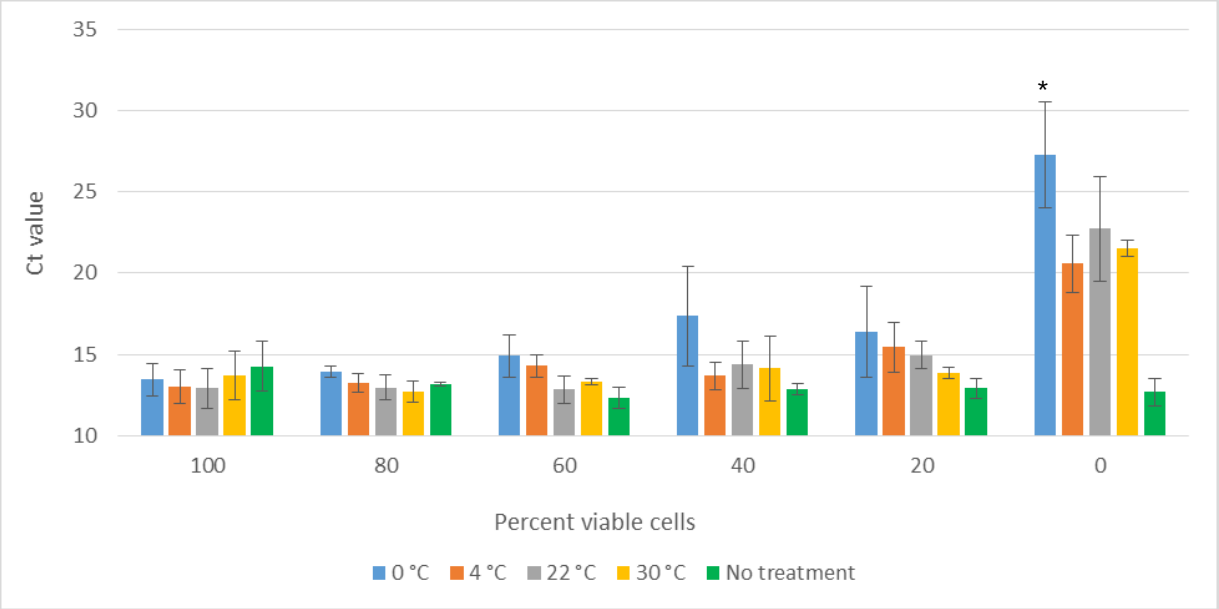

Figure 16. Bars represent the average Ct value of samples exposed to PEMAX dye at different incubation temperatures at different amounts of live *Y. ruckeri* (10^9 CFU mL^{-1}). *n* = 3. * = significant difference from 4, 22 and 30 °C with 0 % live cells ($p < 0.05$). Note, 80, 60, 40 and 20 % live cells not analysed for statistical significance. Error bars represent the SD between replicates.

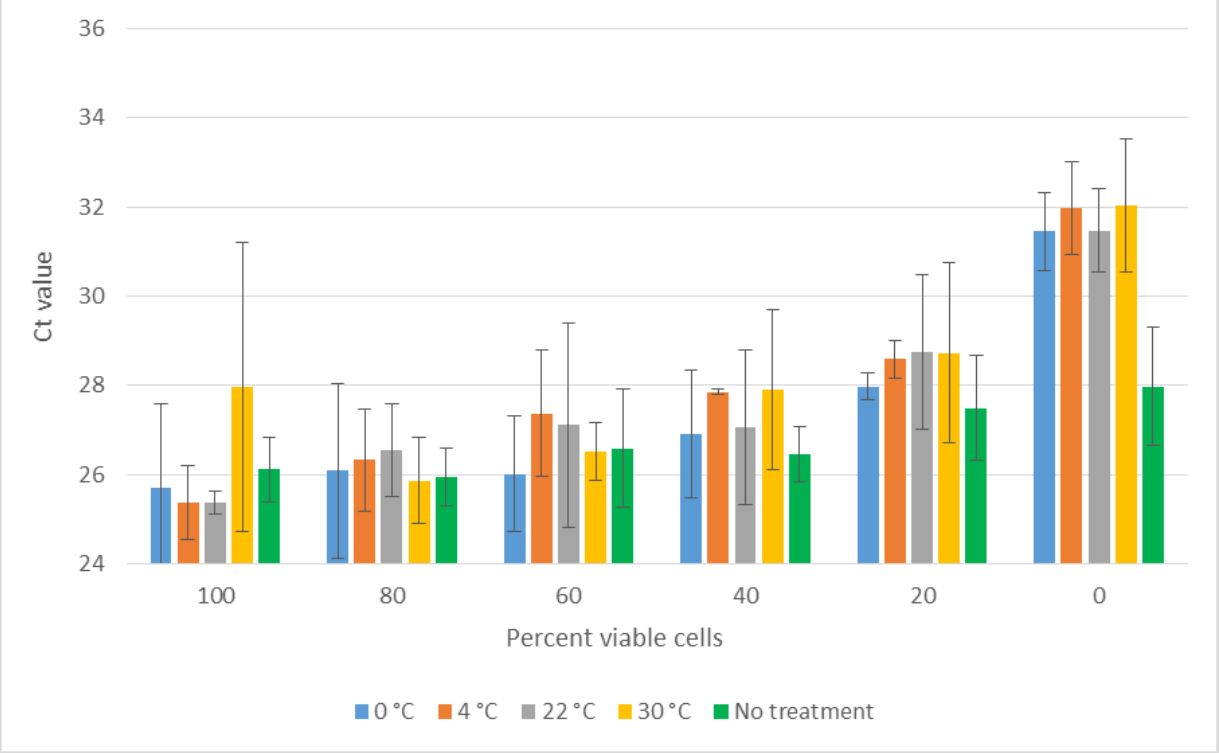

Figure 17. Bars represent the average Ct value of samples exposed to PEMAX dye used at different incubation temperatures with different amounts of live *Y. ruckeri* (10^5 CFU mL^{-1}). *n* = 3. Note, 80, 60, 40 and 20 % live cells not analysed for statistical significance. Error bars represent the SD between replicates.

To ensure the dye had no effect on live cells, statistical analysis of the Ct values was carried out comparing the following groups: live cells, dead cells and controls. Differences were as expected (Table 5).

Table 5: Comparison of the treated cells with the controls – summary of expected results.

Cell type comparison	Expected result	Interpretation
Controls and dead treated cells	$p < 0.05$	Dye is penetrating and binding with dead cell DNA.
Live and dead treated cells	$p < 0.05$	Dye is penetrating and binding with dead cell DNA and not penetrating live cells.
Live treated cells and controls	$p > 0.05$	Dye is not penetrating and binding with live cells.

As the samples incubated on ice revealed a greater differentiation between live and dead cells, this temperature was used in subsequent experiments.

No significant differences between any of the incubation temperatures tested on any bacterial concentrations were observed for EMA v-qPCR (Figures 18 and 19). For EMA, RT was used as incubation temperature in subsequent experiments.

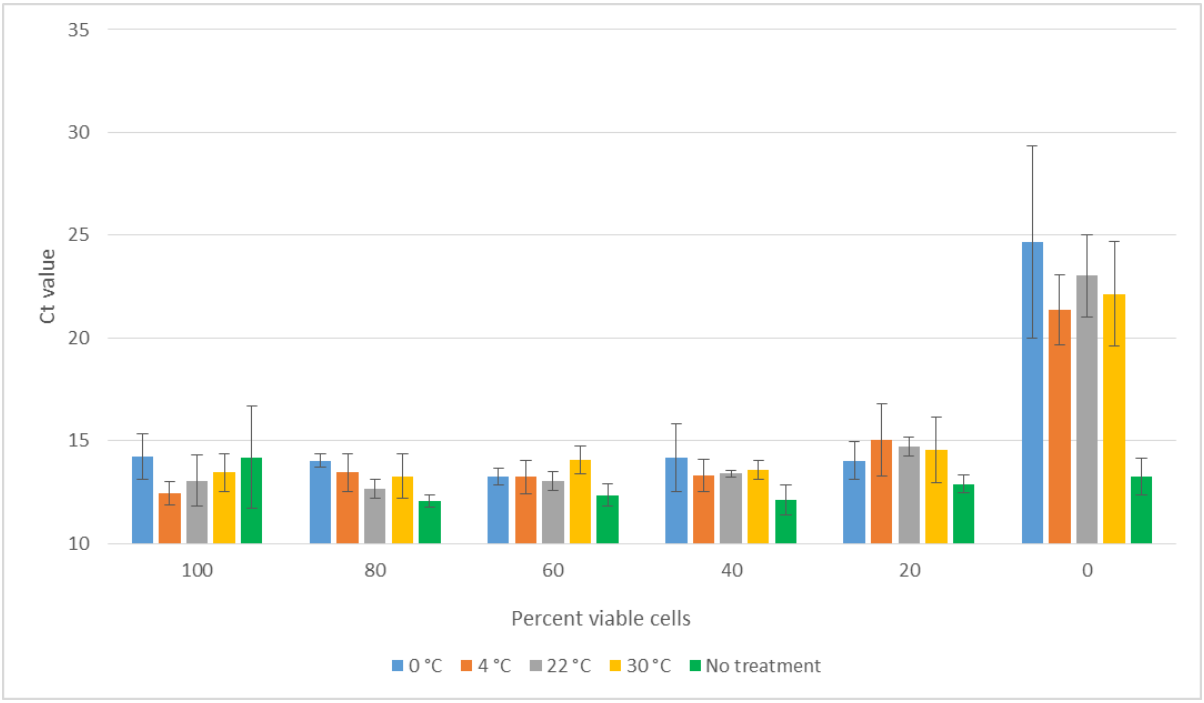

Figure 18. Bars represent the average Ct value for samples exposed to EMA dye at different incubation temperatures with different amounts of live *Y. ruckeri* (10^9 CFU mL^{-1}). n = 3. Note, 80, 60, 40 and 20 % live cells not analysed for statistical significance. Error bars represent the SD between replicates.

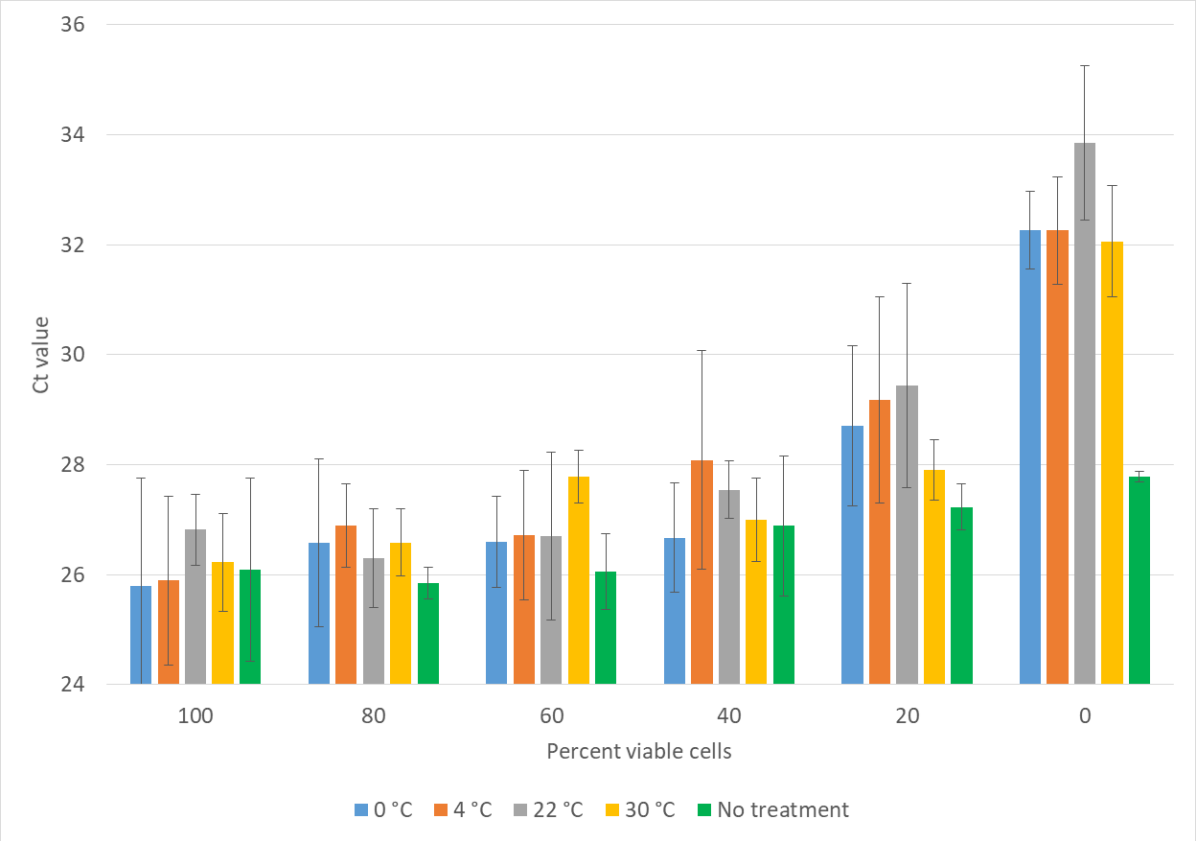

Figure 19. Bars represent the average Ct value for samples exposed to EMA dye at different incubation temperatures with different amounts of live *Y. ruckeri* (10^5 CFU mL^{-1}). *n* = 3. Note, 80, 60, 40 and 20 % live cells not analysed for statistical significance. Error bars represent SD between replicates.

PEMAX gave better results i.e., higher Ct values, than EMA at the higher bacterial concentrations suggesting PEMAX may be a better dye for *Y. ruckeri*. However, these experiments were carried out using different aliquots of *Y. ruckeri* culture therefore, they cannot be directly compared as there will be some degree of variation in the cell volumes in the original aliquot.

Summary: In subsequent *Y. ruckeri* experiments, ice and RT were used as the dye incubation temperatures for PEMAX and EMA, respectively.

Tenacibaculum maritimum

For PEMAX v-qPCR, the incubation temperatures assessed showed there were no significant differences between the results of treated dead cells at any of the bacterial concentrations (Figure 20).

Significant differences were seen when analysing the live cells at 10^8 CFU mL^{-1}. This was seen between 4 and 0 °C, with 0 °C being more efficient. However, there was no significant difference between 0 °C and any of the other temperatures assessed.

A reduced differentiation between live and dead cells that were treated with PEMAX was seen when comparing the lower bacterial concentrations (10^5 and 10^3 CFU mL^{-1}) i.e., a smaller difference in Ct values between live and dead samples.

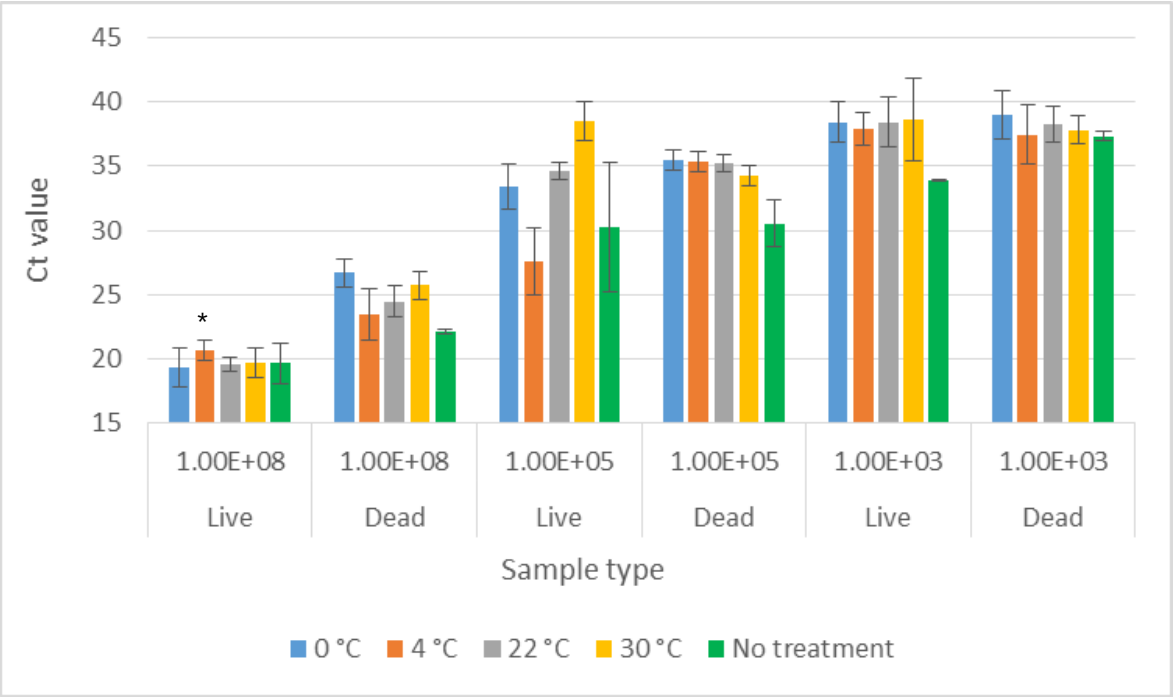

Figure 20. Bars represent the average Ct value of live and dead *T. maritimum* cells (10^8, 10^5, 10^3 CFU mL^{-1}) exposed to PEMAX dye at four different incubation temperatures. $n = 3$. * = significant difference from ice ($p < 0.05$). Error bars represent the SD between replicates.

Comparisons were made between all sample groups (live, dead and control (no treatment)) to ensure the process had no effect on live cells. Results were as expected (see Table 5) for all samples at each bacterial concentration apart from at 10^5 CFU mL^{-1} between the live and dead cells not treated ($p < 0.05$). This is likely due to the increased Ct value of the heat treated cells compared to the live cells because of the degradation of the DNA during the heating step (Karni et al., 2013). At the lower bacterial concentrations (10^5 and 10^3 CFU mL^{-1}), the significance of the results were variable, there was always a significant difference between the live cells with no treatment and dead cells with treatment. However, with the dead cells no treatment and dead cells with treatment or live and dead cell treated there was not a significant difference. This is likely due to the minimal difference in the Ct value observed between the live and dead cells (Figure 18). At 10^5 CFU mL^{-1} there was a significant difference between the live cells not treated and the live cells treated, with the live cells not treated having a lower Ct value (i.e., higher bacterial concentration). This may suggest the dye is penetrating the live cells, however as it was only seen at this cell concentration this is unlikely. The variation of cell clumping between the aliquots is an alternative explanation.

As expected the conventional vPCR for live treated cells produced an amplicon at all incubation temperatures and all bacterial concentrations (Table 6). The vPCR for dead cells produced an amplicon at all incubation temperatures at 10^8 CFU mL^{-1} but this was weaker in intensity than the live treated or not treated samples. Results for the lower bacterial concentrations were variable across all temperatures.

Table 6: Conventional vPCR results for live and dead *T. maritimum* cells at different incubation temperatures for both dyes. Results in the incubation temperature columns indicate the number of replicates out of three that produced an amplicon. Blue = PEMAX; Black = EMA.

			Incubation temperature (°C)				
Bacterial concentration (CFU mL^{-1})	**Dye**	**Live/Dead**	**0**	**4**	**22**	**30**	**Controls (not treated)**
10^8	PEMAX	Live	3	3	3	3	3
10^8	PEMAX	Dead	3	3	3	3	3
10^8	EMA	Live	3	3	3	3	3
10^8	EMA	Dead	3	3	3	3	3
10^5	PEMAX	Live	3	3	3	3	3
10^5	PEMAX	Dead	3	2	3	1	3
10^5	EMA	Live	1	3	3	3	3
10^5	EMA	Dead	3	2	2	1	3
10^3	PEMAX	Live	3	3	3	3	3
10^3	PEMAX	Dead	2	2	1	2	3
10^3	EMA	Live	3	2	2	1	3
10^3	EMA	Dead	1	3	1	1	3

No significant differences were observed in the EMA v-qPCR results for dead cells at any incubation temperature trialled. This was true for all bacterial concentrations treated with EMA dye (Figure 21).

There were no significant differences in EMA treated live cells between the incubation temperatures assessed at high or low bacterial concentrations (10^8, 10^3 CFU mL^{-1}). However, a significant difference was observed at the medium bacterial concentration (10^5 CFU mL^{-1}). This result may have been due to a processing error from the dilutions, pelleting or removal of supernatant. This bacteria is very sticky and prone to clumping which may have resulted in differences when performing dilutions, however as this was only seen at this bacterial dilution in live cells, the result is unlikely to be accurate and was therefore discarded.

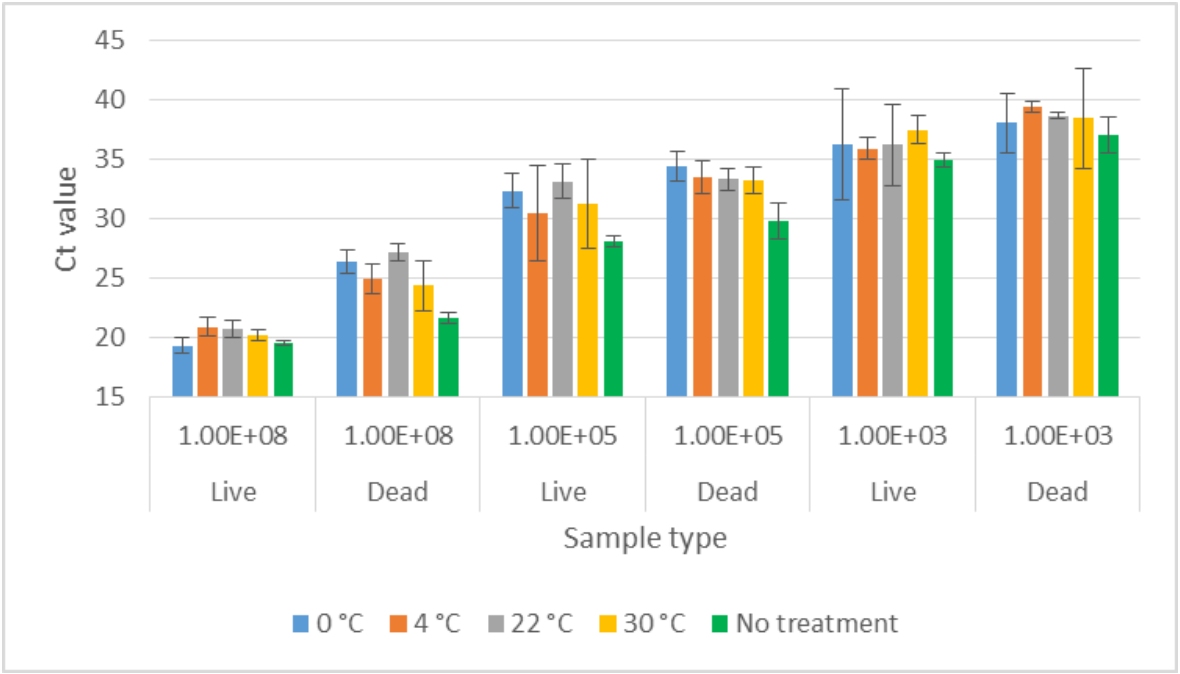

Figure 21. Bars represent the average Ct value of live and dead *T. maritimum* cells (10^8, 10^5, 10^3 CFU mL^{-1}) exposed to EMA at four different incubation temperatures. *n* = 3. Error bars represent the SD between the replicates.

Comparisons between all treatment groups (live, dead and control) were made. At a high bacterial concentration (10^8 CFU mL^{-1}) differences were as expected between the controls (not treated) and dead treated cells and live and dead treated cells (Table 5). At this bacterial concentration there were also differences between the live treated cells and dead not treated and the live not treated and dead not treated samples. As with PEMAX, this is likely due to the increased Ct value of the heat treated cells compared to the live cells due to degradation of the DNA during the heating step (Karni et al., 2013).

At lower bacterial concentrations (10^5 and 10^3 CFU mL^{-1}) there was a significant difference between the live, not treated and dead, treated cells but not with the dead, not treated and dead, treated cells or live and dead treated cells which was not as expected. As previously discussed, this is likely due to the minimal difference in the Ct value observed between the live and dead cells (Figure 20) and is the reason why lower bacterial concentrations should be avoided in the initial validation. There was also a significant difference seen at both of these bacterial concentrations in the live, not treated and live, treated cells. This may suggest EMA is penetrating some of the live cells as the differences are observed at these cell concentrations and not the higher concentration. Alternatively, as there will be some dead cells within the grown up inoculum it may be these cells that are more evident at these lower cell concentrations. Caution should be taken in interpreting results at these lower bacterial concentrations and higher replication in the initial validation should be considered.

The conventional vPCR for EMA treated live cells did not consistently produce an amplicon at the different incubation temperatures and at all bacterial concentrations (Table 6). This result provides further evidence that EMA may have a negative effect on live *T. maritimum* cells. Complete suppression was not observed at any bacterial concentration or incubation temperature.

Summary: incubation at RT (~22 °C) was chosen for both dyes in subsequent *T. maritimum* experiments. The results suggest that EMA may be penetrating the cell wall or membranes of live cells. However, EMA was trialled in further optimisation experiments to confirm these results.

ABV

For the conventional PEMAX vRT-PCR, dead samples at 10^7, 10^5 and 10^4 $TCID_{50}$ did not amplify at any temperature assessed. Samples containing live treated, 10^7, 10^5 $TCID_{50}$ viral concentrations amplified at all temperatures, however the intensity of the amplicons appeared to decrease as the temperature increased (Figure 21). At a concentration of 10^4 $TCID_{50}$, one of three replicates amplified at 4 °C but did not amplify at any other temperature (Table 7). This is most likely because this concentration is below the LOD of the assay.

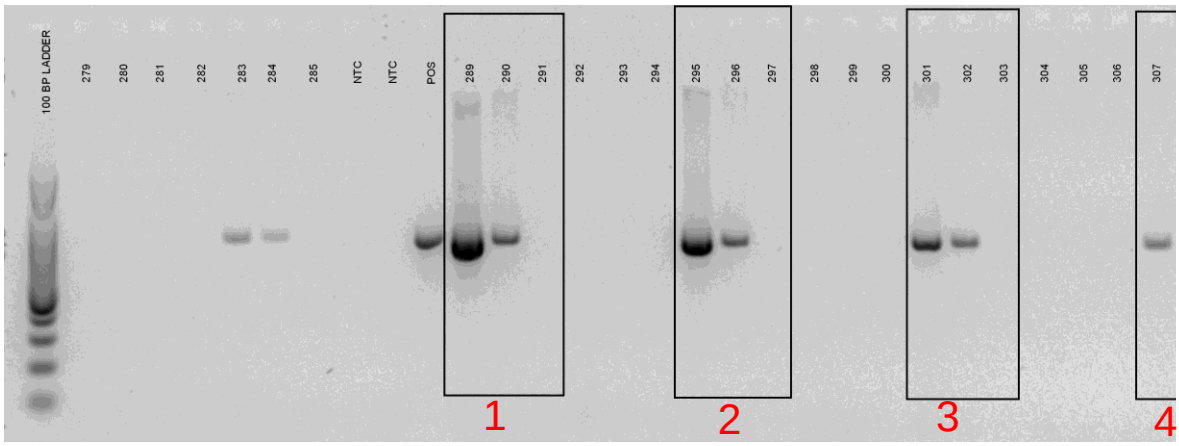

Figure 22. Gel electrophoresis of ABV exposed to PEMAX at 4 different incubation temperatures. Live cells; 10^7, 10^5 and 10^4 $TCID_{50}$ incubated on ice (Box 1), 4 °C (Box 2), 22 °C (Box 3), and Live cells – 10^7 $TCID_{50}$, incubated at 30 °C (Box 4) .

There were no significant differences between controls in all comparisons, however, live, not treated 10^7 and 10^5 $TCID_{50}$ amplified in all replicates and live, not treated 10^4 $TCID_{50}$ only amplified in one of three replicates (Figure 22). Dead, not treated 10^7, 10^5 and 10^4 $TCID_{50}$ amplified in all replicates. Therefore, the live 10^4 $TCID_{50}$ results may either be because is the pathogen concentration is below the LOD of the assay or due to a processing error.

For EMA vRT-PCR, at 10^7 $TCID_{50}$ all dead and treated samples amplified when incubated at 0 °C, two of three amplified when incubated at 4 °C, and no replicates amplified at either 22 or 30 °C. At 10^5 and 10^4 $TCID_{50}$, amplification was not observed at any incubation temperature. The samples containing live treated virus at 10^7 and 10^5 $TCID_{50}$ amplified at all incubation temperatures, although the intensity of the amplicon appeared to decrease as the incubation temperature increased as shown with PEMAX dye (Figure 22). At a viral concentration of 10^4 $TCID_{50}$, amplification was not seen at replicates at any incubation temperature. The controls showed a statistically significant difference between live and dead virus, but not between any other comparisons. All live and dead control samples at all dilutions amplified in the conventional vRT-PCR.

Table 7: Conventional vPCR results for live and dead ABV cells at different incubation temperatures for both dyes. Results in the incubation temperature columns indicate the number of replicates out of three that produced an amplicon. Blue = PEMAX; Black = EMA; Red = a weak amplicon was produced in at least one replicate.

			Incubation temperature (°C)				
Viral concentration($TCID_{50}$)	**Dye**	**Live/Dead**	**0**	**4**	**22**	**30**	**Controls (not treated)**
10^7	PEMAX	Live	3	3	3	3	3
10^7	PEMAX	Dead	0	0	0	3	3
10^7	EMA	Live	3	3	3	3	3
10^7	EMA	Dead	3	2	0	3	3
10^5	PEMAX	Live	3	3	3	3	3
10^5	PEMAX	Dead	0	0	0	1	3
10^5	EMA	Live	3	3	3	3	3
10^5	EMA	Dead	0	0	0	1	3
10^4	PEMAX	Live	1	0	0	3	3
10^4	PEMAX	Dead	0	0	0	2	3
10^4	EMA	Live	0	0	0	1	3
10^4	EMA	Dead	0	0	0	1	3

The comparison of the lower concentration of virus may suggest that EMA and PEMAX are penetrating the live virus and the changes are only noticeable at the lower concentrations. Alternatively the result could be due to the low concentration being at the LOD of the RT-PCR or due to pipetting error. At these lower dilutions it is difficult to interpret optimisation results. As with bacteria, it is recommended to only use a high concentration of virus in the initial optimisation stages. It is unknown if RNA degradation would be seen in naturally infected tissue that contains dead virus.

Summary: Ice (0 °C) incubation was used for both dyes in subsequent ABV experiments. The reduction in intensity of the amplicons with increased temperature was likely due to the RNA degradation as RNA is less stable at higher temperatures.

4.6 OPTIMISING PHOTOPERIOD

Yersinia ruckeri

At 10^9 and 10^5 CFU mL^{-1}, there were no significant differences observed using v-qPCR with live or dead PEMAX treated cells exposed for different photoperiods (Figure 23). When comparing controls with live and dead treated cells, the differences were as expected with no suggestion of PEMAX penetrating live cells (as per Table 5).

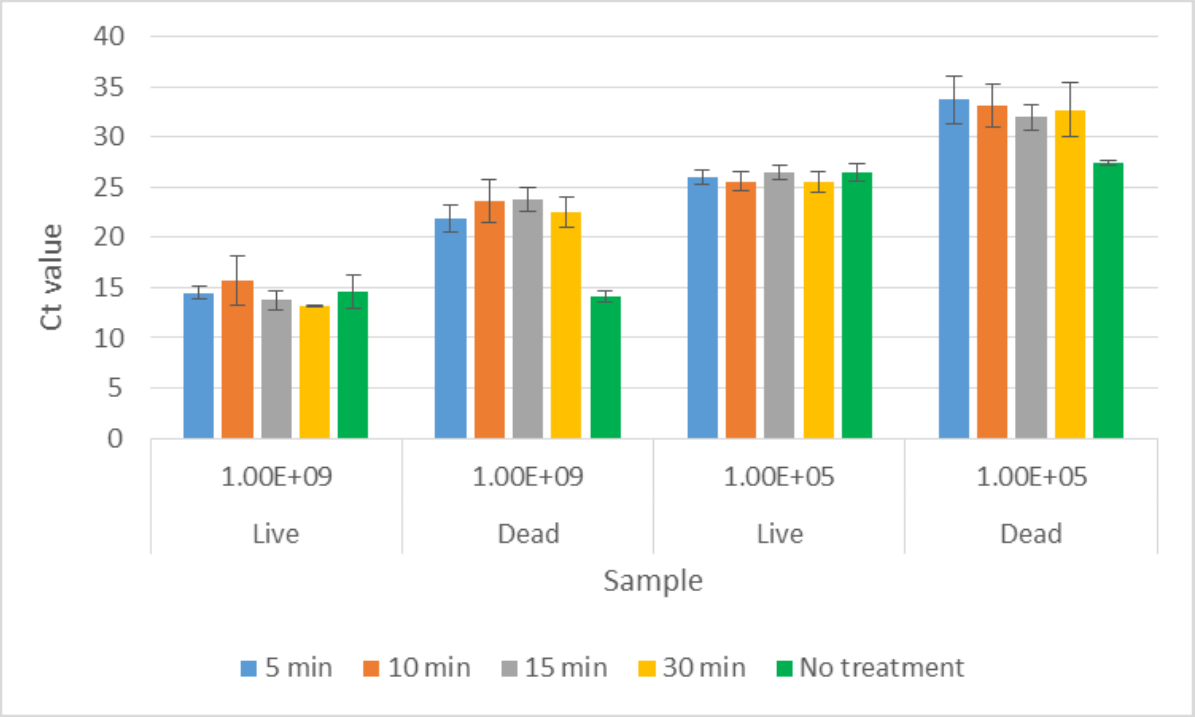

Figure 23. Bars represent average Ct value of live and dead *Y. ruckeri* at 10^9 or 10^5 CFU mL^{-1} exposed to PEMAX at four different photoperiods. *n* = 3. Error bars represent the SD between replicates.

Different photoperiods using EMA v-qPCR resulted in no significant differences observed at 10^8 CFU mL^{-1}, however differences were observed at 10^5 CFU mL^{-1} with 15 min being the least effective. No differences were observed for live treated cells at both bacterial concentrations and photoperiods (Figure 24).

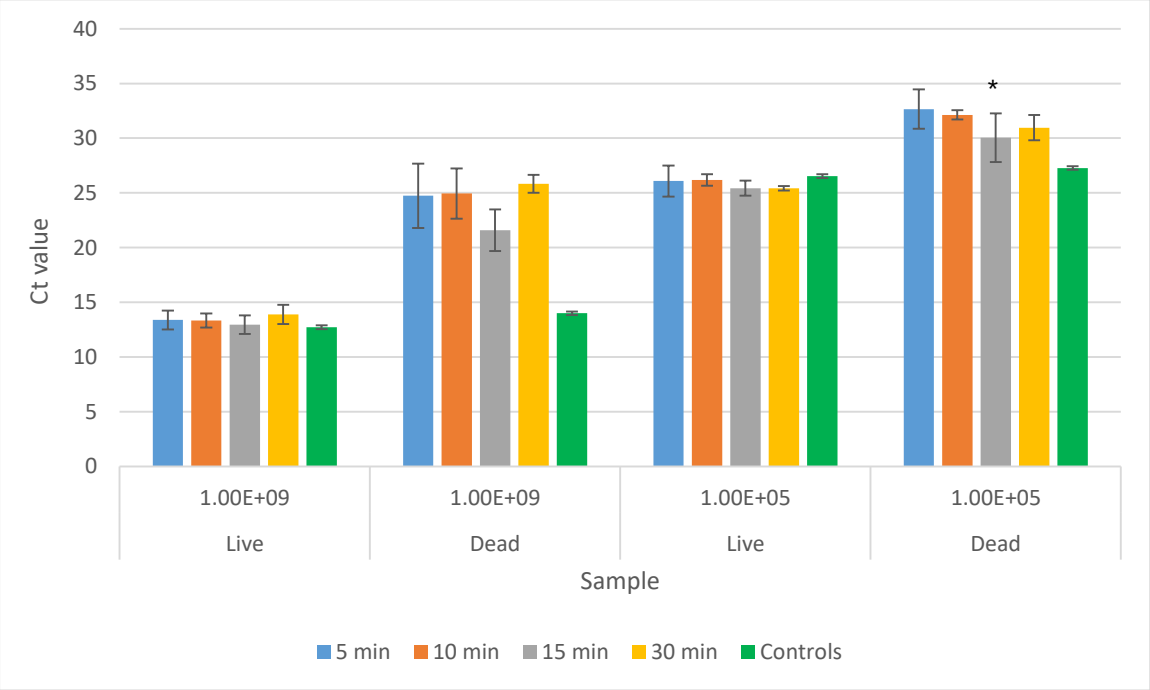

Figure 24. Bars represent average Ct value of live and dead *Y. ruckeri* at 10^9 or 10^5 CFU mL^{-1} exposed to EMA at four different photoperiods. *n* = 3. Error bars represent the SD between replicates. * = significant difference from other dead cells at 10^5 CFU mL^{-1} ($p < 0.05$).

When comparing the different groups (controls, live and dead cells) the differences were as expected with no suggestion of EMA penetrating live cells as per Table 5.

Summary: based on the results from the high *Y. ruckeri* concentration, the default setting of 15 min was used for both dyes in subsequent experiments.

Tenacibaculum maritimum

For the PEMAX q-vPCR on dead cells, significant differences were observed at the 10^8 and 10^3 CFU mL^{-1} following exposure to different photoperiods (Figure 25). For the higher concentrations of bacteria, 15 min was the more effective photoperiod while 10 min was the least effective photoperiod for the lower bacterial concentrations.

The PEMAX v-qPCR for live cells at 10^8 and 10^3 CFU mL^{-1} resulted in no significant differences between photoperiods evaluated. However, a 10 min photoperiod was the most effective time for 10^5 CFU mL^{-1} (Figure 25).

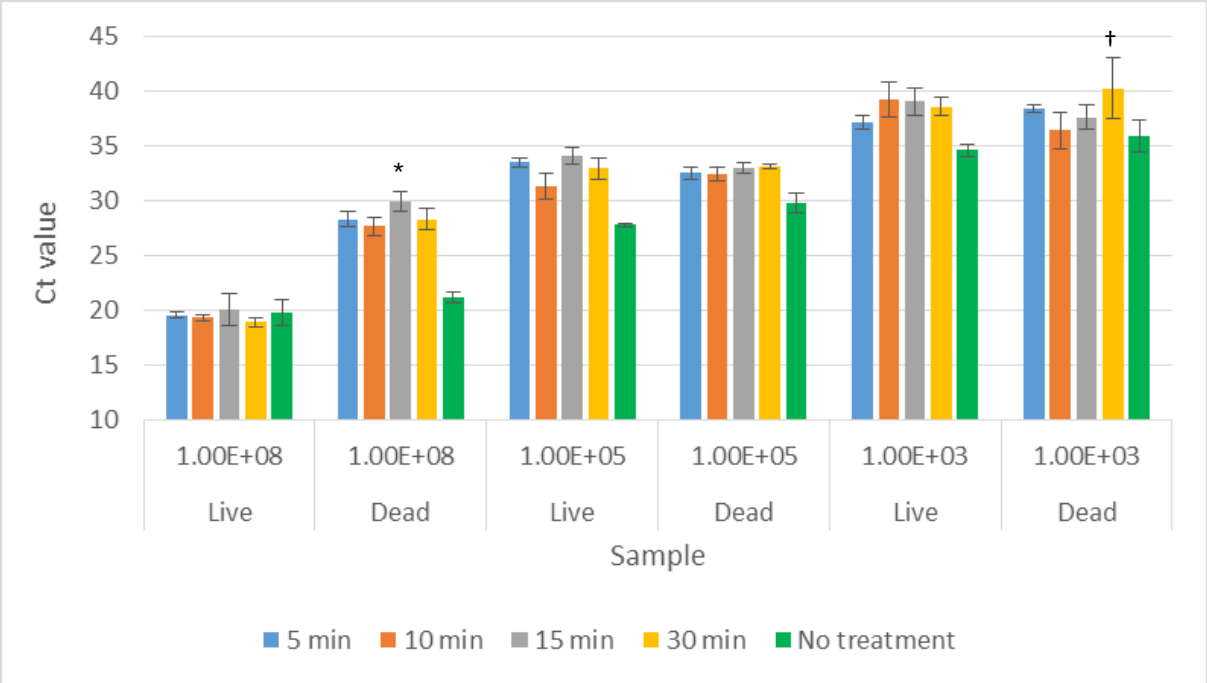

Figure 25. Bars represent average Ct values of *T. maritimum* (10^8, 10^5, and 10^3 CFU mL^{-1}) exposed to PEMAX and four different photpoeriods. $n = 3$. * = significant difference from dead cells at 10^3 CFU mL^{-1} ($p < 0.05$), †= significant different from 10 min dead cells at 10^5 CFU mL^{-1} ($p < 0.05$). Error bars represent the SD between replicates.

Comparisons between the groups (live, dead and controls) were as expected as per Table 5. However, when analysing the bacterial concentrations separately, the differences were significant as expected for the high bacterial concentrations, but were variable for the lower bacterial concentrations.

For the EMA v-qPCR on dead cells, longer photoperiods were more effective at 10^8 CFU mL^{-1}. No significant differences were observed at the lower bacterial concentrations.

For live cells, a longer exposure had a negative impact at the lower bacterial concentrations (Figure 26).

Summary: For subsequent *T. maritimum* experiments, a 15 min photoperiod was used for both dyes. This decision was based on the results derived from the high concentration of bacteria.

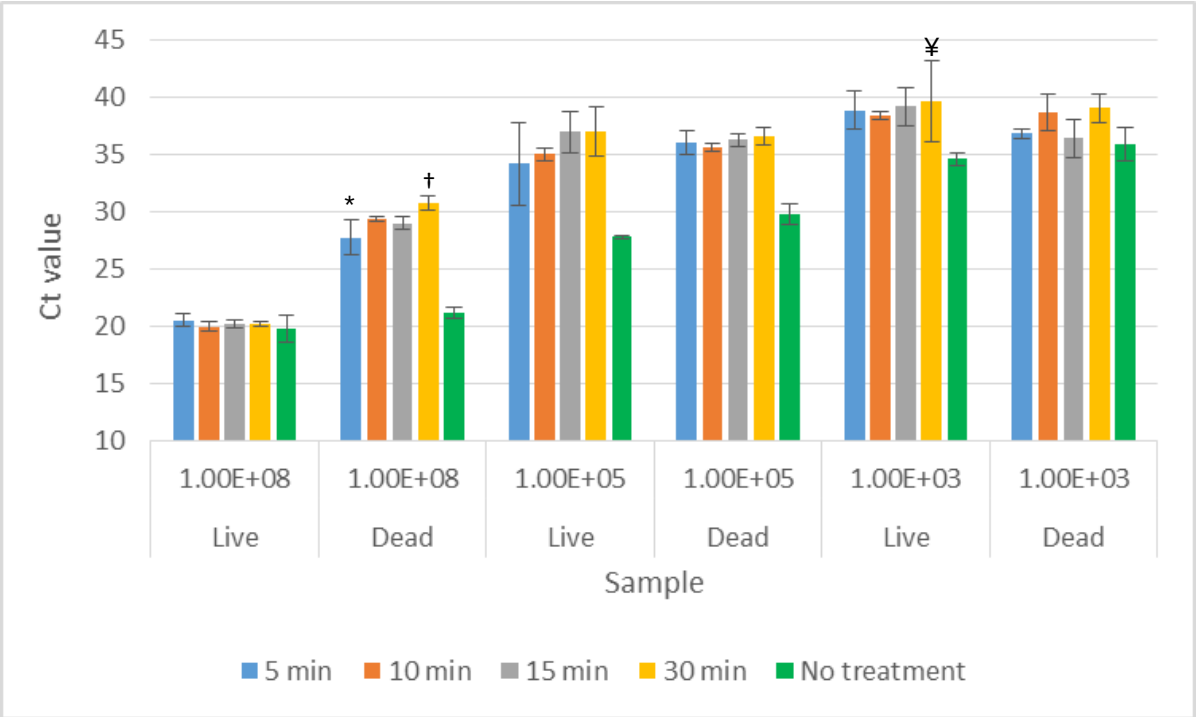

Figure 26. Bars represent average Ct values of *T. maritimum* (10^8, 10^5, and 10^3 CFU mL^{-1}) exposed to EMA and four different photoperiods. $n = 3$. * = significant difference from 10 and 30 min ($p < 0.05$), † = significant difference from 15 min ($p < 0.05$), ¥ = significant difference from 15 min ($p < 0.02$). Error bars represent SD between replicates.

Comparisons between the groups (live, dead and controls) were as expected as per Table 5. However as shown in Figure 25, there is a large difference between the live, not treated sample and all the treated samples at both 10^5 and 10^3 CFU mL^{-1}. This may be a genuine result and that EMA is penetrating some live cells, or as discussed above it could be an artefact of there being some dead cells in the original inoculum, a dilution discrepancy, an issue with the sample clumping, or because the test at these concentrations is at the LOD of the assay.

Conventional PCR showed amplification at all exposure times at the high bacterial concentration for both dyes. At the lower bacterial concentrations both live and dead samples amplified variably. All controls amplified (Table 8).

Table 8: Conventional vPCR for different photoperiods at three bacterial concentrations of *T. maritimum* with both dyes. Results in the exposure time columns indicate the number of replicates out of three that produced an amplicon. Black text = EMA, Blue text = PEMAX.

			Exposure time (min)				
Bacterial concentration (CFU mL^{-1})	**Dye**	**Live/Dead**	**5**	**10**	**15**	**30**	**Controls (not treated)**
10^8	PEMAX	Live	3	3	3	3	3
10^8	PEMAX	Dead	3	3	3	3	3
10^8	EMA	Live	3	3	3	3	3
10^8	EMA	Dead	3	3	3	3	3
10^5	PEMAX	Live	3	3	2	3	3
10^5	PEMAX	Dead	2	2	1	1	3
10^5	EMA	Live	3	2	3	2	3
10^5	EMA	Dead	1	2	2	2	3
10^3	PEMAX	Live	3	2	2	3	3
10^3	PEMAX	Dead	1	1	3	1	3
10^3	EMA	Live	2	0	2	1	3
10^3	EMA	Dead	2	2	1	2	3

4.7 ASSESSING THE USE OF RESUSPENSION BUFFERS

Yersinia ruckeri

For the PEMAX v-qPCR, the resuspension buffer containing broth was significantly less effective than all other buffers assessed. This may be due to the turbidity of the sample which has been previous shown to influence v-PCR sensitivity (Gedalanga & Olsen, 2009).

All concentrations of Triton X-100 assessed and 0.01 % SD resulted in significantly improved results for dead cells compared to 0.3 % SD. For PEMAX, 0.1 % Triton was used for subsequent experiments as it gave the highest Ct result overall, but equally 0.01 % SD, 0.5 % and 1 % Triton X-100 could have been used (Figure 27). The buffers did not appear to affect the results from live cells.

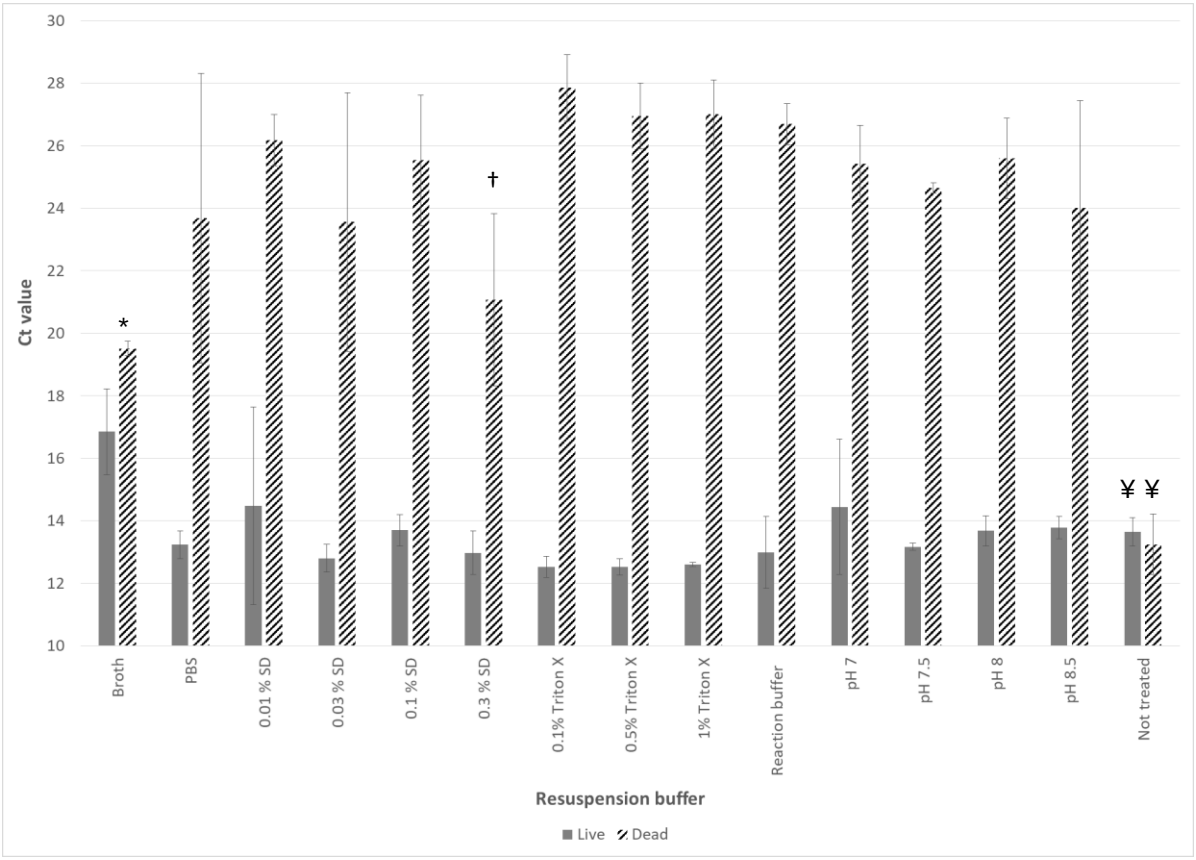

Figure 27. Bars represent average Ct value of live and dead *Y. ruckeri* cells (10^9 CFU mL^{-1}) in different reaction buffers exposed to PEMAX dye. *n* = 3. * = significant difference from 0.01 % SD, 0.1 % SD, 0.1, 0.5 and 1 % Triton-X-100, pH 7, 7.5 and 8, Reaction buffer ($p < 0.05$). † = significant difference from 0.01 % SD, 0.1, 0.5 and 1 % Triton X-100 ($p < 0.05$). ¥ = significant difference from dead treated cells ($p < 0.01$). Error bars represent the SD between replicates.

Conventional vPCR using any resuspension buffer did not result in complete suppression of dead treated cells for either dye. For the EMA v-qPCR on dead cells, no significant differences were observed between any of the buffers evaluated. For the EMA v-qPCR on live cells, a significant difference was observed in broth compared to all other buffers and 1 % Triton X-100 was shown to be less efficient than 0.1 % Triton X-100. The differences observed for live cells in broth could be due to the membrane composition as *Y. ruckeri* was growing well in this media. Rapidly dividing *Escherichia coli* cells have been reported to be negatively affected by EMA (Gedalanga & Olsen, 2009). This also indicates Triton X-100 used at a higher concentration may compromise the cell wall or membrane allowing EMA to penetrate (Figure 28). Interestingly, this did not seem to be the case for PEMAX. As PEMAX has lower concentrations of EMA they may be at a level that the effect is not detected and that PMA (at a higher level in the dye) is not penetrating the live cells.

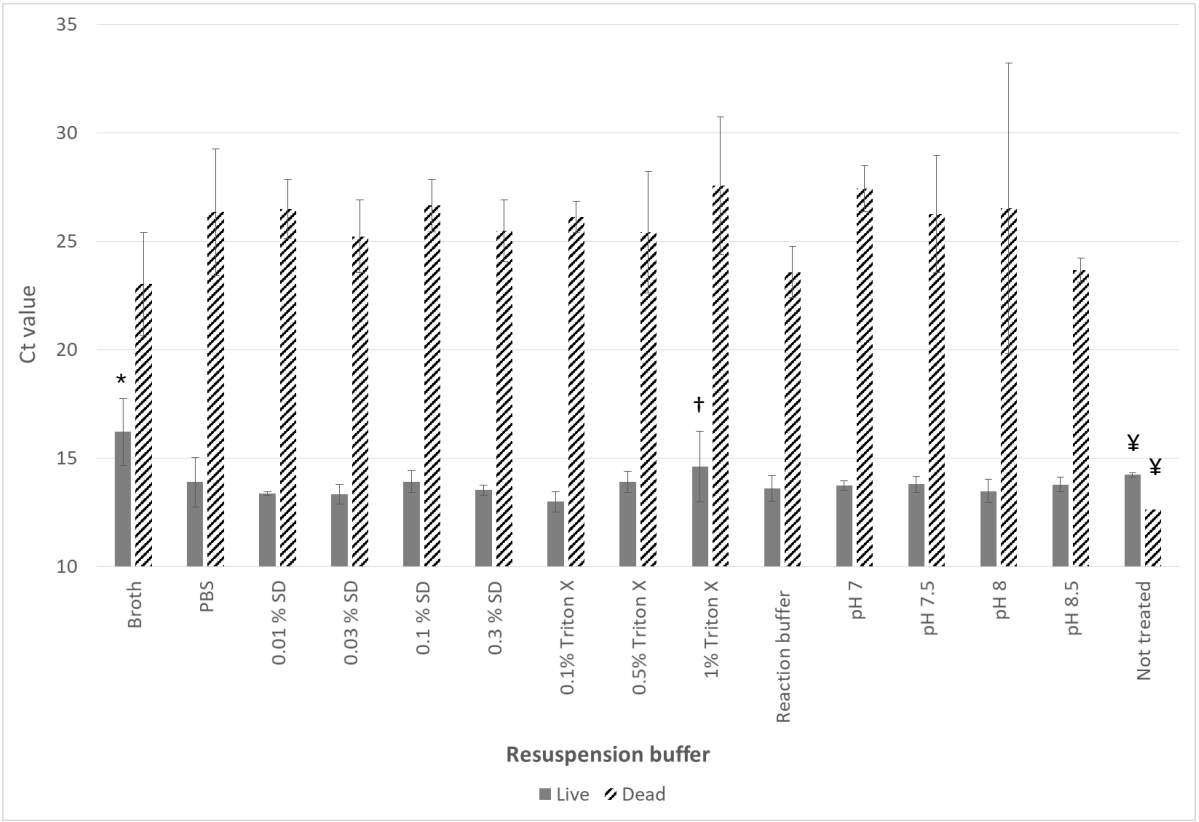

Figure 28. Bars represent the average Ct value of live and dead *Y. ruckeri* cells (10^9 CFU mL^{-1}) in different reaction buffers exposed to EMA. *n* = 3. * = significant difference from 0.01, 0.03, 0.1, 0.3 % SD, 0.1, 0.5 % Triton X-100, PBS, pH 7, 7.5, 8, and 8.5, reaction buffer ($p < 0.01$). † = significant difference from 0.1 % Triton X ($p < 0.05$). ¥ = significant difference from dead treated cells ($p < 0.01$). Error bars represent the SD between replicates.

Analysis between the groups (live treated, dead treated and controls) were as expected (see Table 5).

Results from conventional vPCR for both dyes show that full suppression of amplicons was not consistent within replicates. Some buffers resulted in an observed loss of intensity of the amplicons compared to the live cells and the controls (red numbers in Table 9).

Table 9: Conventional vPCR for resuspension buffers trialled with PEMAX and EMA on 10^8 CFU mL^{-1} of *Y. ruckeri*. The number in the resuspension buffer columns equals the number of replicates out of three that produced an amplicon. The red numbers indicate at least two of three replicates produced a weak or very weak amplicon when compared to the live or control samples.

		Resuspension buffer														
Dye	**Live/Dead**	**Broth**	**PBS**	**0.01% SD**	**0.03% SD**	**0.1% SD**	**0.3% SD**	**0.1% Triton X**	**0.5% Triton X**	**1% Triton X**	**Reaction Buffer**	**pH 7**	**pH 7.5**	**pH 8**	**pH 8.5**	**Controls**
EMA	**Live**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**
EMA	**Dead**	**3**	**3**	**3**	**2**	**3**	**3**	**3**	**2**	**3**	**3**	**3**	**3**	**3**	**3**	**3**
PEMAX	**Live**	**3**	**3**	**2**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**
PEMAX	**Dead**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**2**	**3**	**3**	**3**	**3**	**3**	**3**	**3**

Summary: PEMAX was the optimal dye for *Y. ruckeri* as previous experiments indicated that EMA was impacting the live cells. Triton X-100 at a final concentration of 0.1 % was used as the reaction buffer as overall it gave the highest Ct value for dead cells exposed to PEMAX and did not appear to impact live cells. PBS can be used as a resuspension buffer for EMA treated *Y. ruckeri* samples.

Tenacibaculum maritimum

For the PEMAX v-qPCR on dead cells, broth and seawater resulted in significantly less efficiency than other buffers (Figure 29). The reduced effectiveness of seawater is not surprising as the salt concentration of this sample matrix has been found to reduce the effectiveness of vPCR. This is likely to be due to the competition between the positively charged salt ions and the dye molecules binding to the negatively charged DNA (Shi et al., 2011).

Similarly, broth and seawater were shown to be significantly less efficient than the other buffers for the EMA v-qPCR on dead cells. In addition, pH 7 was shown to be significantly less efficient than 0.01 % SD, 0.1 % Triton X-100, PBS, pH 8 and pH 8.5 (Figure 30).

For v-qPCR of both dyes, the buffers assessed did not appear to have a negative effect on live cells.

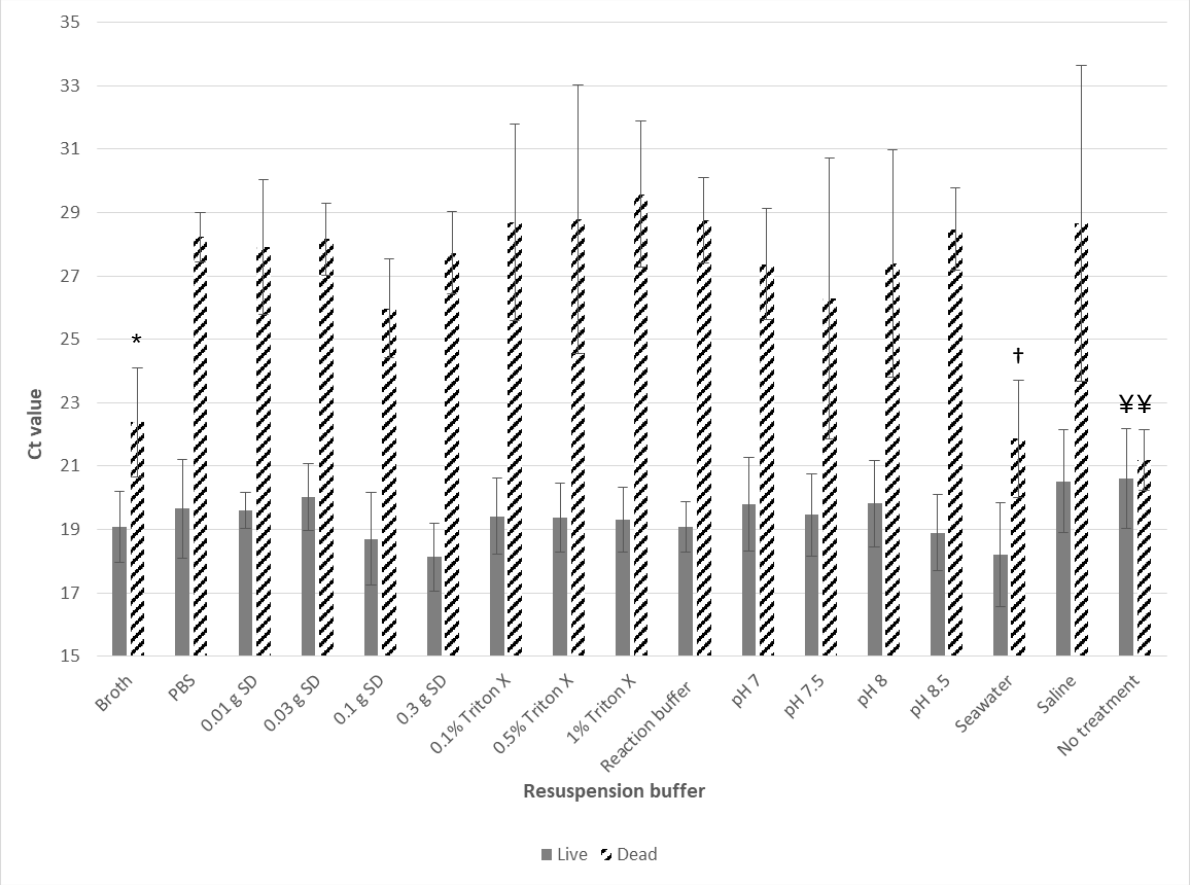

Figure 29. Bars represent the average Ct value of live and dead *T. maritimum* (10^8 CFU mL^{-1}) in different reaction buffers exposed to PEMAX. n = 3. * = significant difference from 0.01, 0.03, 0.3 % SD, 0.1 % Triton X-100 ($p < 0.05$). † = significant difference from 0.1, 0.03, 0.03 % SD, 0.1 % Triton X-100 ($p < 0.05$). ¥ = significant difference from dead treated cells ($p < 0.01$). Error bars represent the SD between replicates.

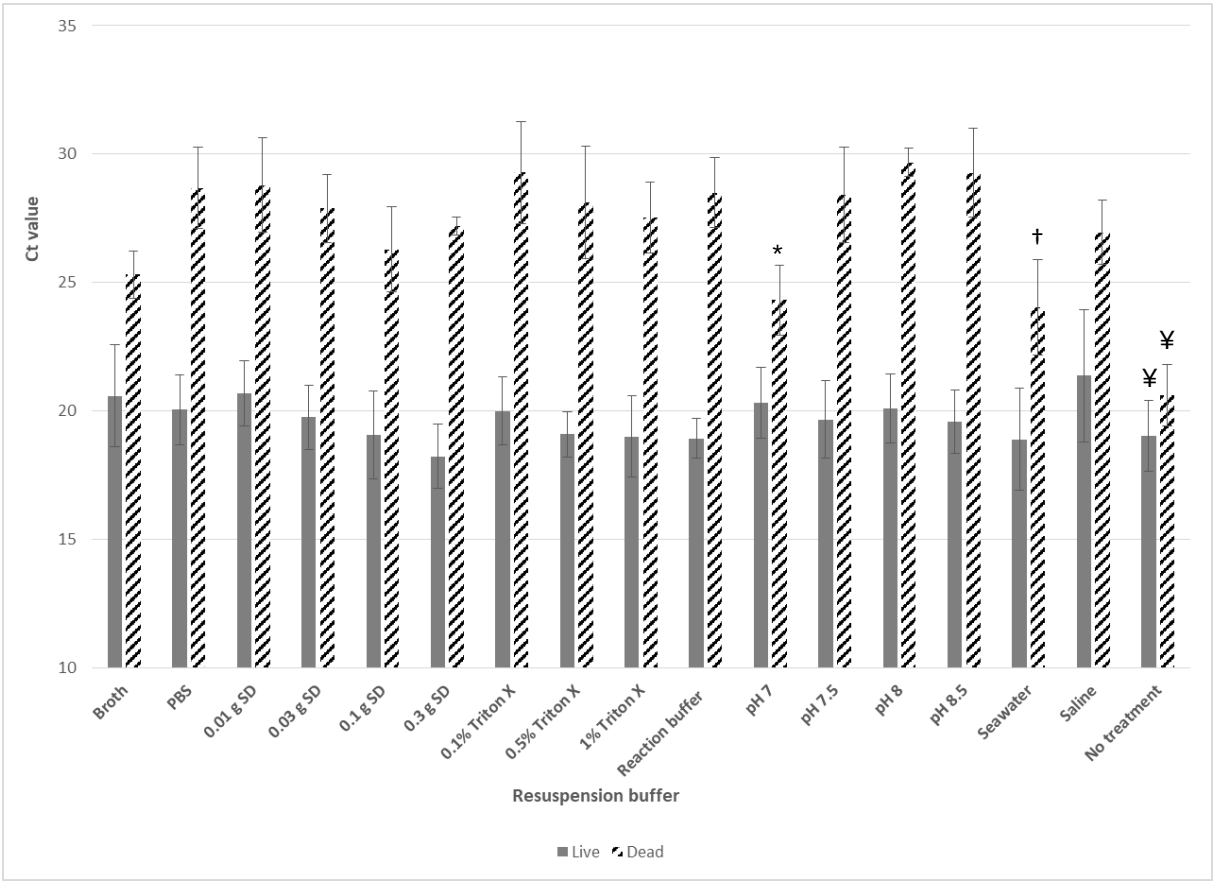

Figure 30. Bars represent the average Ct value of live and dead *T. maritimum* (10^8 CFU mL^{-1}) in different reaction buffers exposed to EMA. $n = 3$. * = significant difference from 0.01 % SD, 0.1 % Triton X-100 ($p < 0.05$). † = significant difference from 0.1 % SD, 0.1 % Triton X-100 ($p < 0.05$). ¥ = significant difference from dead treated cells ($p < 0.01$). Error bars represent SD between replicates.

Analysis between the groups (live treated, dead treated and controls) were as expected (see Table 5).

Conventional vPCR did not consistently show full suppression for the high concentration of bacteria using either dye (Table 10). Triton X-100 used at both 0.1 % and 0.5 % and 0.01 % SD had no amplification in at least one replicate with PEMAX dye, however as these buffers showed no significant difference when compared in the v-qPCR they were not selected for further optimisation.

Table 10: Conventional vPCR for resuspension buffers evaluated with PEMAX and EMA on 10^7 CFU mL^{-1} of *T. maritimum*. The number in the resuspension buffer columns equals the number of replicates that produced an amplicon. The red numbers indicate at least two of three replicates produced a weak or very weak amplicon when compared to the live or control samples.

		Resuspension buffer																
Dye	**Live/Dead**	**Broth**	**PBS**	**0.01% SD**	**0.03% SD**	**0.1% SD**	**0.3% SD**	**0.1% Triton X**	**0.5% Triton X**	**1% Triton X**	**Reaction Buffer**	**pH 7**	**pH 7.5**	**pH 8**	**pH 8.5**	**Seawater**	**Saline**	**Controls**
EMA	**Live**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**
EMA	**Dead**	**3**	**3**	**2**	**3**	**3**	**3**	**2**	**2**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**
PEMAX	**Live**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**
PEMAX	**Dead**	**3**	**3**	**2**	**3**	**3**	**3**	**1**	**2**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**

4.8 ASSESSMENT OF DOUBLE DYE EXPOSURE

Yersinia ruckeri

For the PEMAX v-qPCR, on dead cells, the double dose of 100 µM was found to be the most effective treatment (Figure 31).

For the PEMAX v-qPCR on live cells, significant differences were observed between 2 x 100 µM and 1 x 100 µM, and 2 x 50 µM and 1 x 100 µM. The two treatments that showed an increase in the Ct value were the ones that were pelleted. This result may be an artefact of the double dye procedure as there will be some loss of bacteria when pelleting and removing supernatant. Further, as these samples had Ct values similar to the live control, the difference in Ct value could also be due to the variation of starting material in the aliquots.

Analysis between the groups (live treated, dead treated and controls) were as expected (see Table 5).

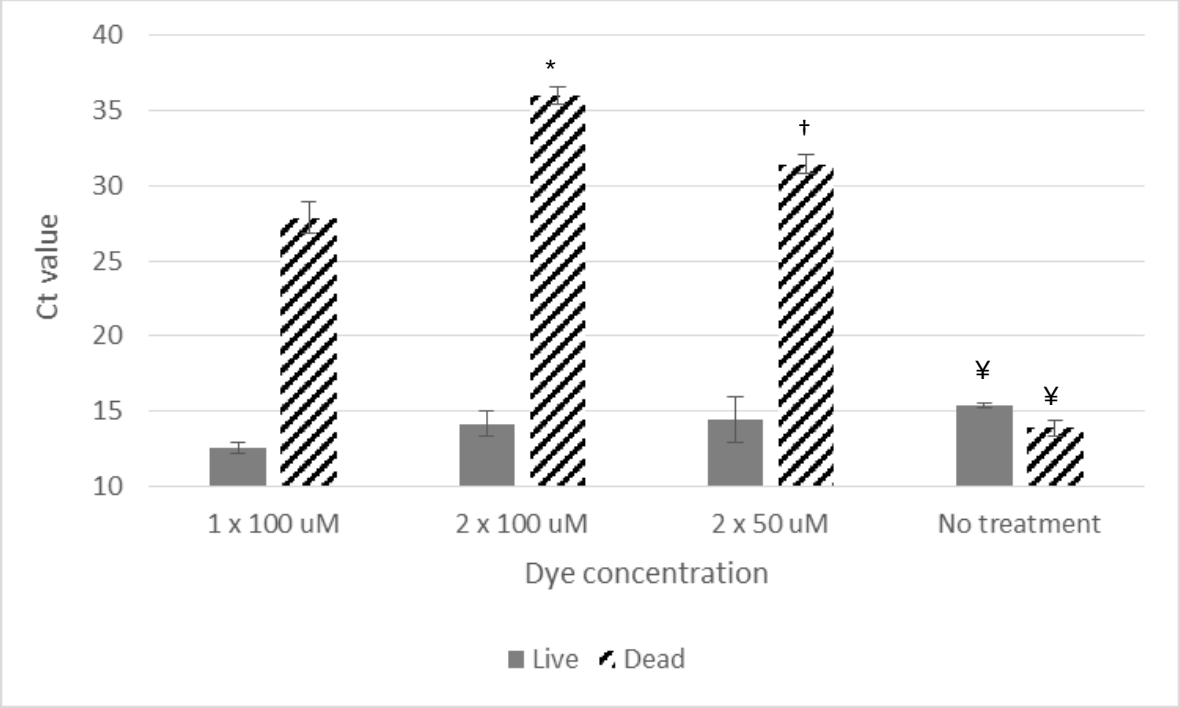

Figure 31. Bars represent average Ct values of live and dead *Y. ruckeri* cells (10^9 CFU mL^{-1}) exposed to different PEMAX treatment regimes. *n* = 3. * = significant difference from 1 x 100 µM and 2 x 50 µM ($p < 0.01$). † = significant difference from 1 x 100 µM ($p < 0.01$). ¥ = significant difference from dead treated cells ($p < 0.01$). Error bars represent SD between replicates.

For the EMA v-qPCR no significant differences were seen between the dye treatments on live or dead cells.

Analysis between the groups (live treated, dead treated and controls) were as expected (see Table 5).

These results show that while the double dye exposure with EMA treatment did not improve the vPCR, it did not have an adverse effect on live cells (Figure 32).

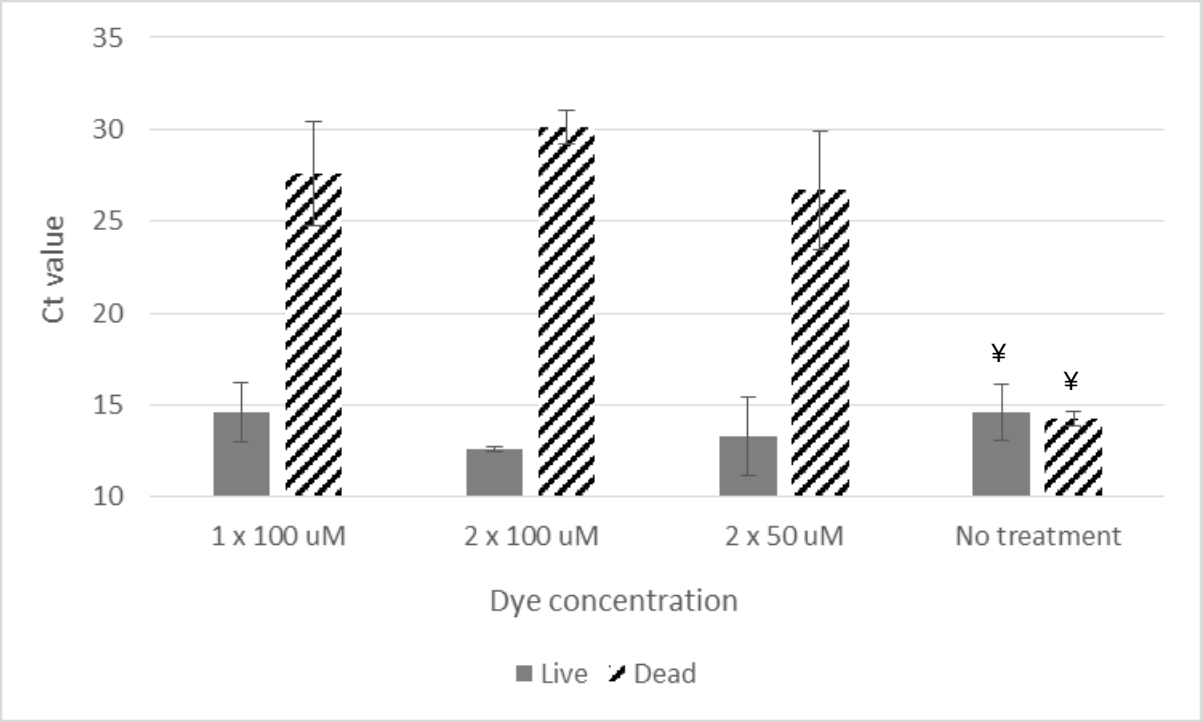

Figure 32. Bars represent average Ct values of live and dead *Y. ruckeri* (10^9 CFU mL^{-1}) cells exposed to different EMA treatment regimes. n = 3. ¥ = significant difference from dead treated cells ($p < 0.01$). Error bars represent SD between replicates.

Summary: A PEMAX dye treatment of 2 x 100 µM was used for subsequent *Y. ruckeri* experiments. The double EMA dye treatment did not result in any improvement, therefore this regime was not subsequently used.

Tenacibaculum maritimum

For the PEMAX v-qPCR carried out on dead cells, a single treatment was found to be more effective than the double dye treatment ($p < 0.01$) (Figure 33).

For the PEMAX v-qPCR carried out on live treated cells, the single treatment had a significantly higher Ct value than the double dye treatment ($p < 0.01$).

Analysis between the groups (live treated, dead treated and controls) were as expected (see Table 5).

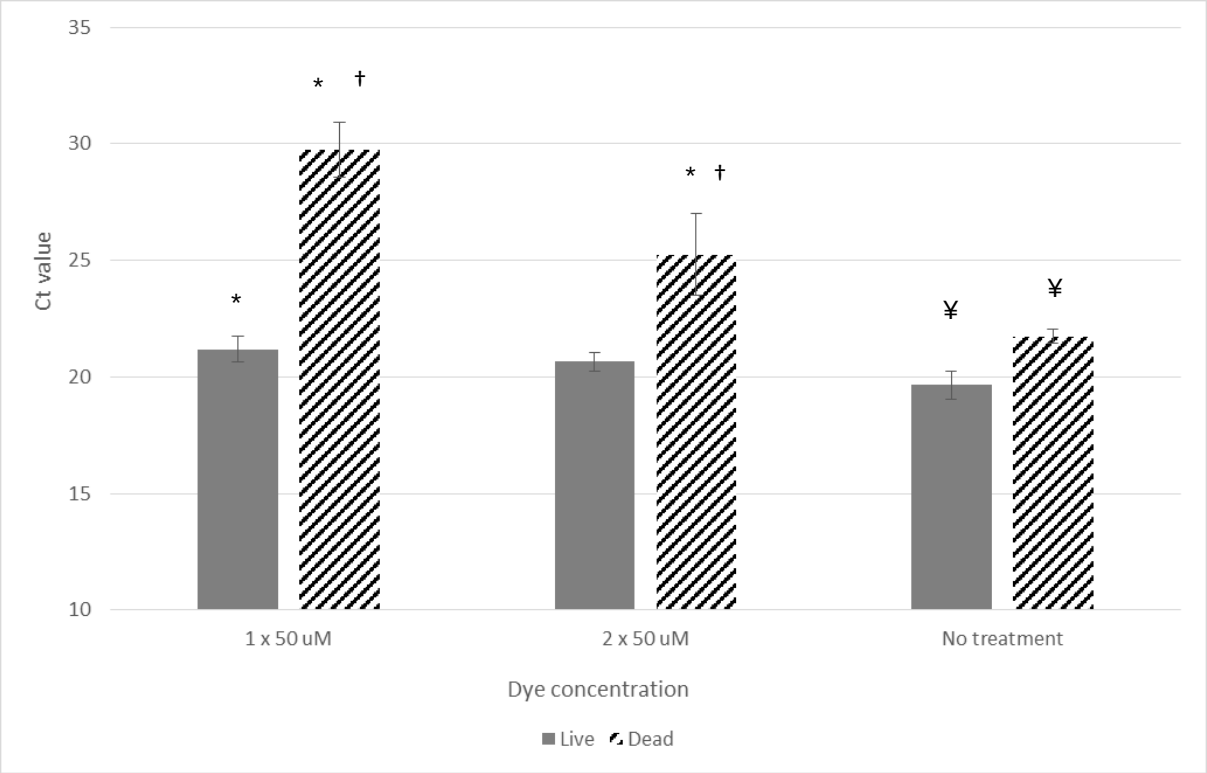

Figure 33. Ct values of live (block colour bar) and dead (lined bar) *T. maritimum* cells exposed to no PEMAX, 1 x and 2 x PEMAX treatment. *n* = 3. * = significant difference from 2 x 50 µM ($p < 0.01$). † = significant difference from the live treated cells ($p < 0.01$). ¥ = significant difference from dead treated and live treated cells ($p < 0.01$).

For the EMA v-qPCR, the different dye treatments resulted in no differences between live and dead cells (Figure 34).

When comparing the groups (controls, live and dead), significant differences were seen as expected (see Table 5). There was also a significant difference shown between live treated and dead, not treated and live, not treated and dead, not treated samples. This has been discussed previously and is likely an artefact of the heat treatment step degrading the DNA.

Summary: a single treatment of PEMAX at a concentration of 50 uM was used for *T. maritimum* in subsequent experiments.

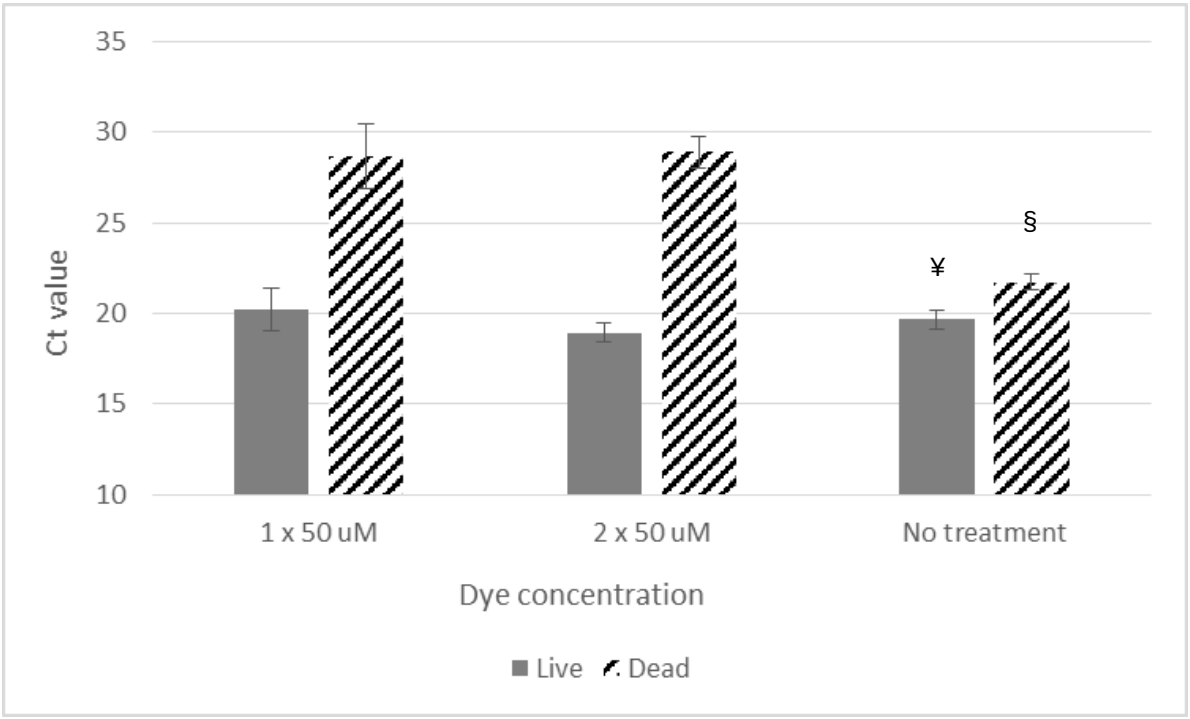

Figure 34. Bars represent the average Ct values of live and dead *T. maritimum* cells exposed to different EMA treatment regimes. *n* = 3. ¥ = significant difference from dead treated cells ($p < 0.01$). § = significant difference from dead treated, live treated and live not treated ($p < 0.01$). Error bars represent the SD between replicates.

4.9 ASSESSMENT OF LONGER DYE INCUBATION (*YERSINIA RUCKERI*)

No significant differences were observed between the PEMAX v-qPCR results from dead cells suggesting that longer incubation times do not increase the efficiency of vPCR for *Y. ruckeri*.

There was a significant difference observed in the PEMAX v-qPCR results on live cells where 30 and 45 min incubations gave the higher Ct values than the other times (Figure 35). As these Ct values are similar to those of the controls, they are more likely a result of differences in the starting material rather than being due to an adverse effect of the dye.

When comparing the groups (controls, live and dead), significant differences were seen as expected (see Table 5). However, there was also a significant difference between live treated and dead not treated samples. As discussed above, this is likely due to the variation in the inoculum when aliquoting or could also be due to the heat treatment degrading the DNA.

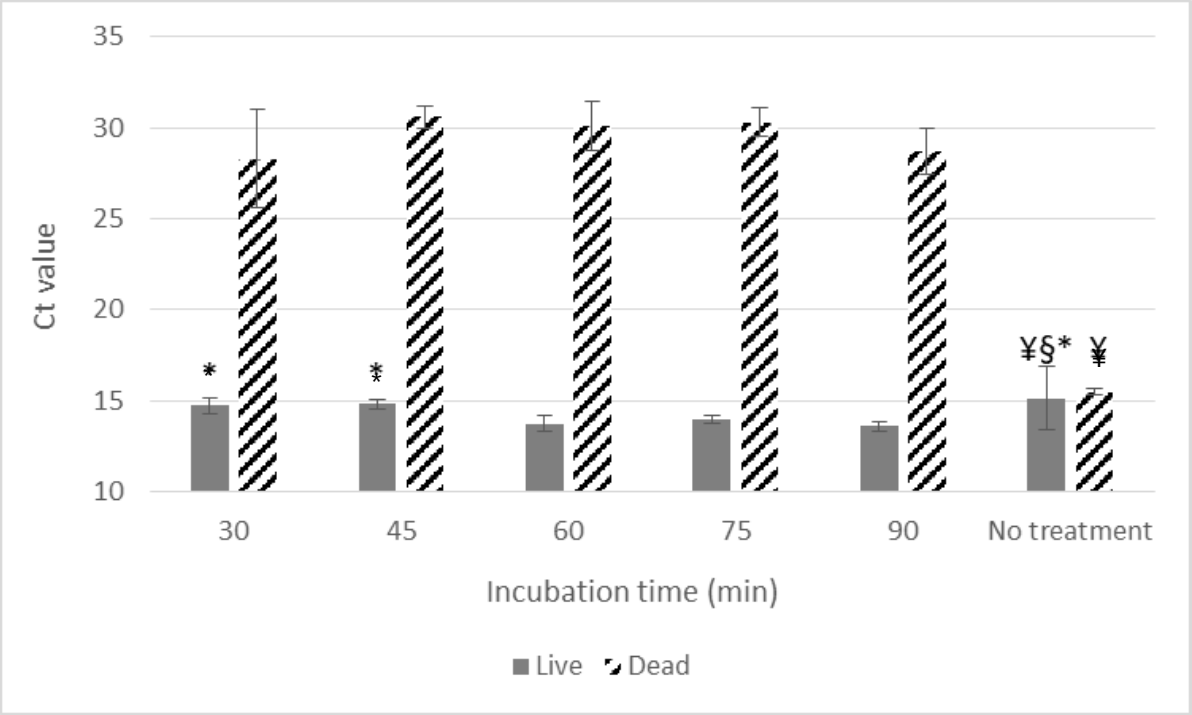

Figure 35. Effect of longer PEMAX incubation time on live and dead *Y. ruckeri* cells. *n* = 3. ¥ = significant difference from dead treated samples ($p < 0.01$). § = significant difference from dead treated samples and dead control ($p < 0.01$). * = significant difference from live treated cells at: 60, 75, and 90 min ($p < 0.01$).

Conventional PCR showed amplification of all live and dead samples. The amplicons were observed to be weaker in the samples containing dead cells however these were not empirically measured. Complete suppression was not seen with any sample (Table 11).

Table 11: Conventional vPCR for incubation times trialled with PEMAX (high concentration of *Y. ruckeri* only). The number in the incubation time columns equals the number of replicates that produced an amplicon. The red numbers indicate at least two or three replicates produced a weak or very weak amplicon when compared to the live or control samples.

	Incubation time (min)					
Live/Dead	**30**	**45**	**60**	**75**	**90**	**Controls**
Live	3	3	3	3	3	3
Dead	3	3	3	3	3	3

Summary: as a longer incubation time did not improve the efficiency of vPCR for *Y. ruckeri*, an incubation time of 15 min was used in subsequent experiments (as optimised in section 4.4).

4.10 ASSESSMENT OF A "DOUBLE TUBE" METHOD (*YERSINIA RUCKERI*)

Using the double tube method did not improve the effectiveness of PEMAX v-qPCR for *Y. ruckeri*. Further, it did not eliminate false positive results from the DNA debris on the tube.

This experiment also demonstrated the variations based on the plastic-ware used. The dark tube was not a specialised vPCR tube and was shown to be the least effective as most debris appeared to remain

in the tube (Figures 36 and 37). The higher the Ct value for the DNA debris tubes (1st tube) the lower the amount of DNA on the tube. The results show that the live cells remain in the tube more than the dead cells, and that less DNA remained on the vPCR tubes compared to the dark tubes.

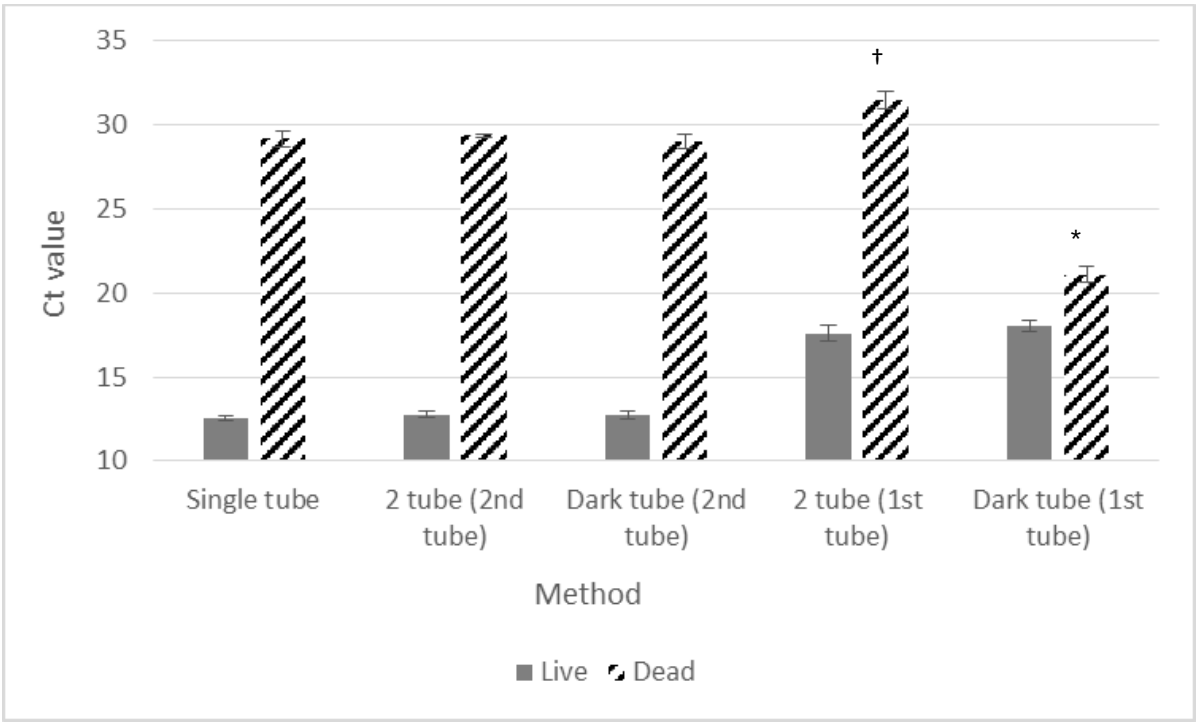

Figure 36. Ct values from the different tube methods for live and dead *Y. ruckeri* cells at 10^8 CFU mL^{-1}. Note: 1st tube samples are DNA debris, the higher the Ct value, the less DNA debris remains in the tube. $n = 3$. * = significant difference from all other dead samples ($p < 0.01$), † = significant difference from all other dead samples ($p < 0.01$).

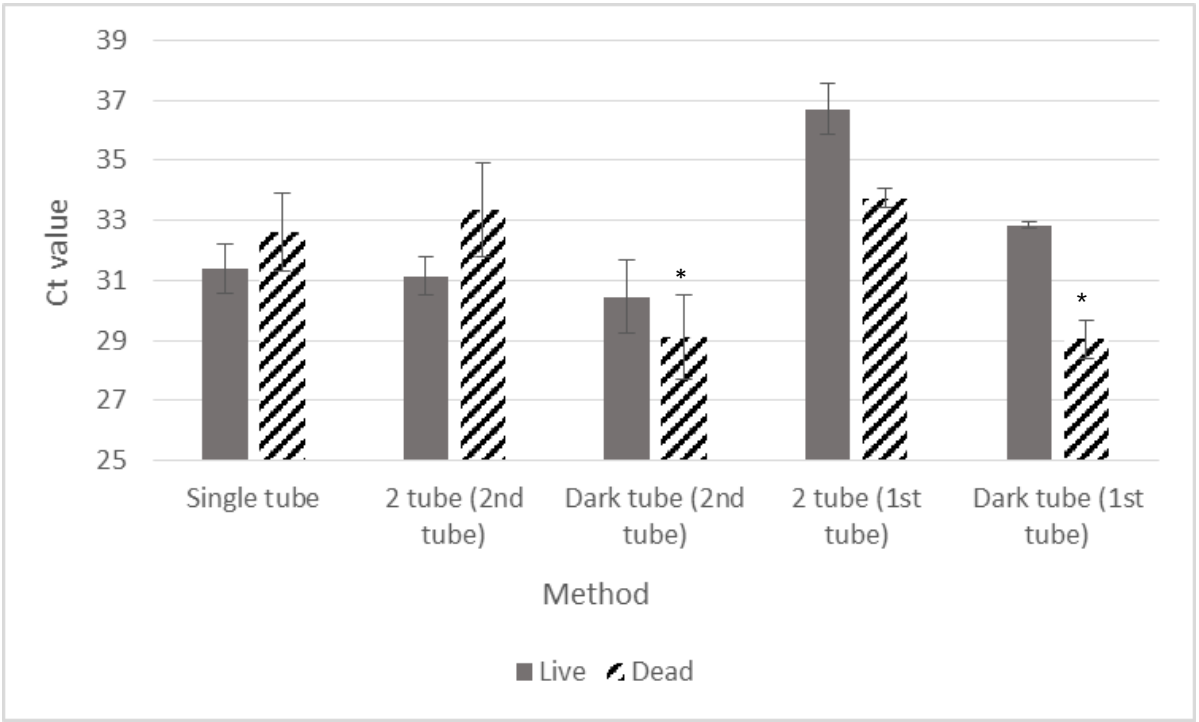

Figure 37. Ct values from the different tube methods for live and dead *Y. ruckeri* cells at 10^5 CFU mL^{-1} bacterial concentration. Note: 1st tube samples are DNA debris, the higher the Ct value, the less DNA debris remains in the tube. $n = 3$. * = significant difference from all other methods ($p < 0.01$).

Summary: The double tube method did not improve effectiveness of the *Y. ruckeri* assays and was not used in subsequent experiments.

4.11 ASSESSMENT OF PELLET WASHING (*YERSINIA RUCKERI*)

Washing dead treated cells twice yielded the least efficient PEMAX v-qPCR results, while excluding the washing step retained the most efficiency (Figure 38).

No significant differences were observed between the washing steps for the PEMAX v-qPCR on live treated cells.

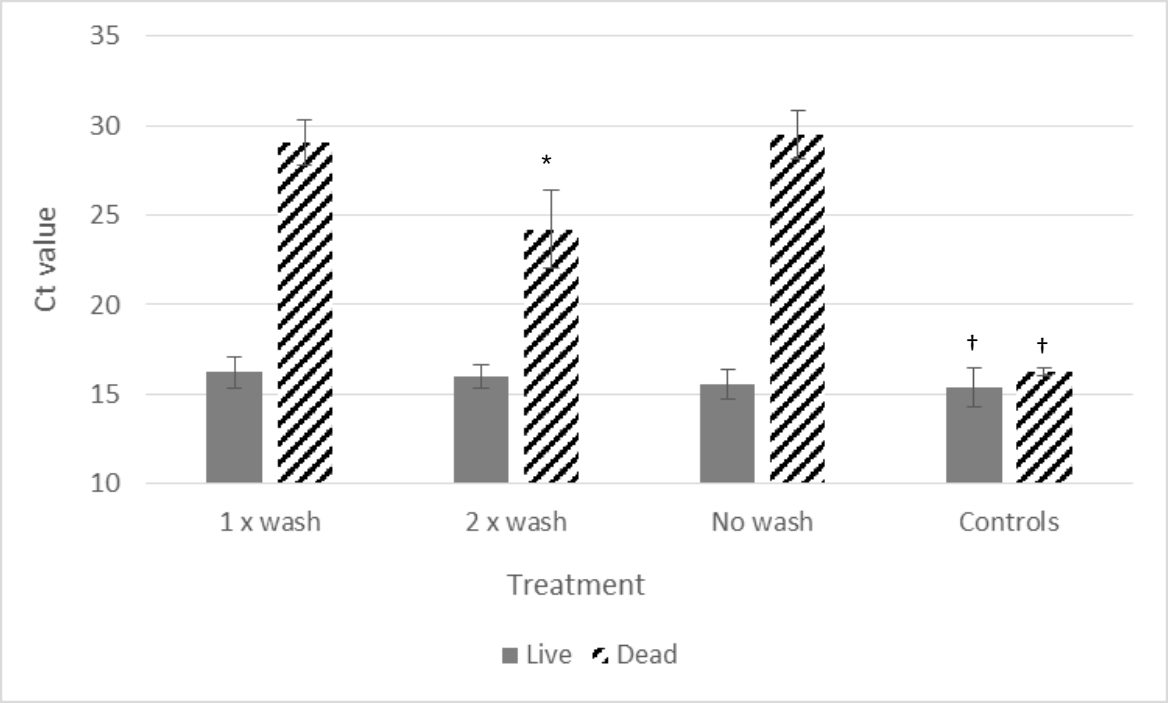

Figure 38. Bars represent average Ct value of live and dead *Y. ruckeri* cells following different pellet washing regimes. $n = 3$. * = significant difference between 1 x wash and no wash ($p < 0.01$). † = significant difference from dead treated samples ($p < 0.01$). Error bars represent SD between replicates.

Summary: Washing steps did not improve effectiveness of the *Y. ruckeri* assay and were not used in subsequent experiments.

4.12 OPTIMAL ASSAY CONDITIONS FOR ALL PATHOGENS

Table 12 provides a summary of the optimised parameters that were used for each pathogen in the subsequent experiments.

Table 12: Optimised parameters for each pathogen.

Variable	*Yersinia ruckeri*	*Tenacibaculum maritimum*	ABV
LOD	10^4 CFU mL^{-1}	10^3 CFU mL^{-1}	10^4 $TCID_{50}$
Inactivation method	99 °C for 10 min	99 °C for 10 min	99 °C for 20 min
Dye*	PEMAX	PEMAX	PEMAX
Dye concentration	2 x 100 µM	50 µM	100 µM
Dye treatment time*	15 min	15 min	15 min
Incubation temperature	0 °C	22 °C	0 °C
Photoperiod*	15 min, 100 % intensity	15 min, 100 % intensity	15 min, 100 % intensity
Resuspension buffer	0.1 % Triton X	0.85% saline	PBS

*could be removed for future optimisation experiments

4.13 APPLICATION TO HIGH-THROUGHPUT

High-throughput (PAUL) testing reduced the sample volume to 100 µL (due to the well volume of the 96-well plate). The volume in the regular vPCR tubes (PhAST Blue) were also reduced to 100 µL and the dye volume was adjusted accordingly.

Tenacibaculum maritimum

No significant differences were observed in the Ct values for the v-qPCR or conventional vPCR between the two platforms (Figure 39, Table 13).

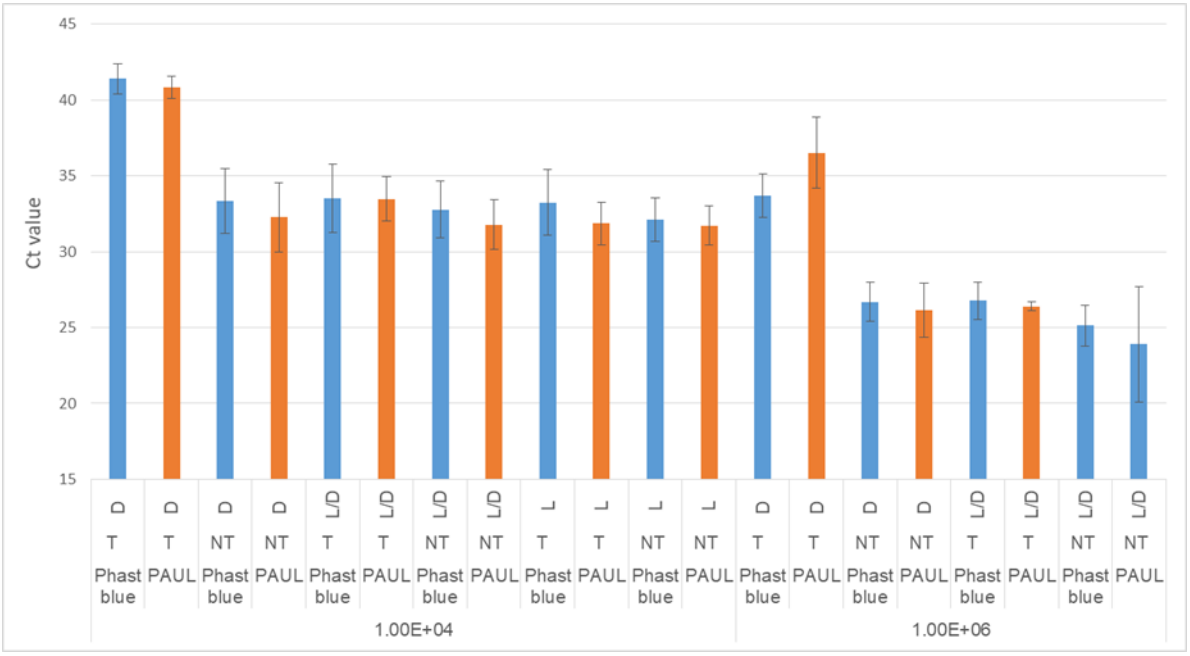

Figure 39. Ct values from the 2 platforms; PhAST blue and PAUL on treated and not treated live and dead *T. maritimum* cells. *n* = 3. L = Live cells; D = Dead cells; L/D = Live/Dead mix of cells.

Table 13: Conventional vPCR results for the 2 platforms; PhAST blue and PAUL on treated and not treated live and dead *T. maritimum* cells. The number in the "PhAST blue" and "PAUL" columns indicate the number of samples that produced an amplicon.

Bacterial concentration (CFU mL^{-1})	Dye treated	Sample	PhAST blue	PAUL
10^3	Yes	Dead 100 %	0/9	0/9
	No	Dead 100 %	3/3	3/3
	Yes	Live 50 %	9/9	9/9
	No	Live 50 %	3/3	3/3
	Yes	Live 100 %	9/9	9/9
	No	Live 100 %	3/3	3/3
10^5	Yes	Dead 100 %	0/9	0/9
	No	Dead 100 %	3/3	3/3
	Yes	Live 50 %	9/9	9/9
	No	Live 50 %	3/3	3/3

ABV

No significant differences were observed in the conventional vRT-PCR results between the two platforms. For both platforms, amplification was not present in samples containing dead virus. This result is consistent with observations from previous experiments and is likely due to the degradation of dead RNA in tissue. Samples containing live virus amplified in both platforms (Table 14).

Table 14: Conventional RT-vPCR results for the 2 platforms; PhAST blue and PAUL on treated and not treated live and dead ABV virus. The number in the "PhAST blue" and "PAUL" columns indicate the number of samples that produced an amplicon.

Viral concentration ($TCID_{50}$)	Dye treated	Sample	PhAST blue	PAUL
10^4	Yes	Dead 100 %	0/9	0/9
	No	Dead 100 %	0/3	3/3
	Yes	Live 50 %	9/9	9/9
	No	Live 50 %	3/3	3/3
	Yes	Live 100 %	9/9	9/9
	No	Live 100 %	3/3	3/3

4.14 PROTOCOL FOR PATHOGEN SPIKED TISSUE

Yersinia ruckeri

At 10^4 CFU mL^{-1} in a matrix of kidney tissue, PEMAX v-qPCR results for 0 % live cells (100 % dead) were on average of 4.35 Ct value higher than those with 100 % live cells. The Ct value of samples gradually increased as the percentage of dead cells increased (Figure 40). The correlation coefficient was -0.85. This concentration of live bacteria was at the LOD of the assay.

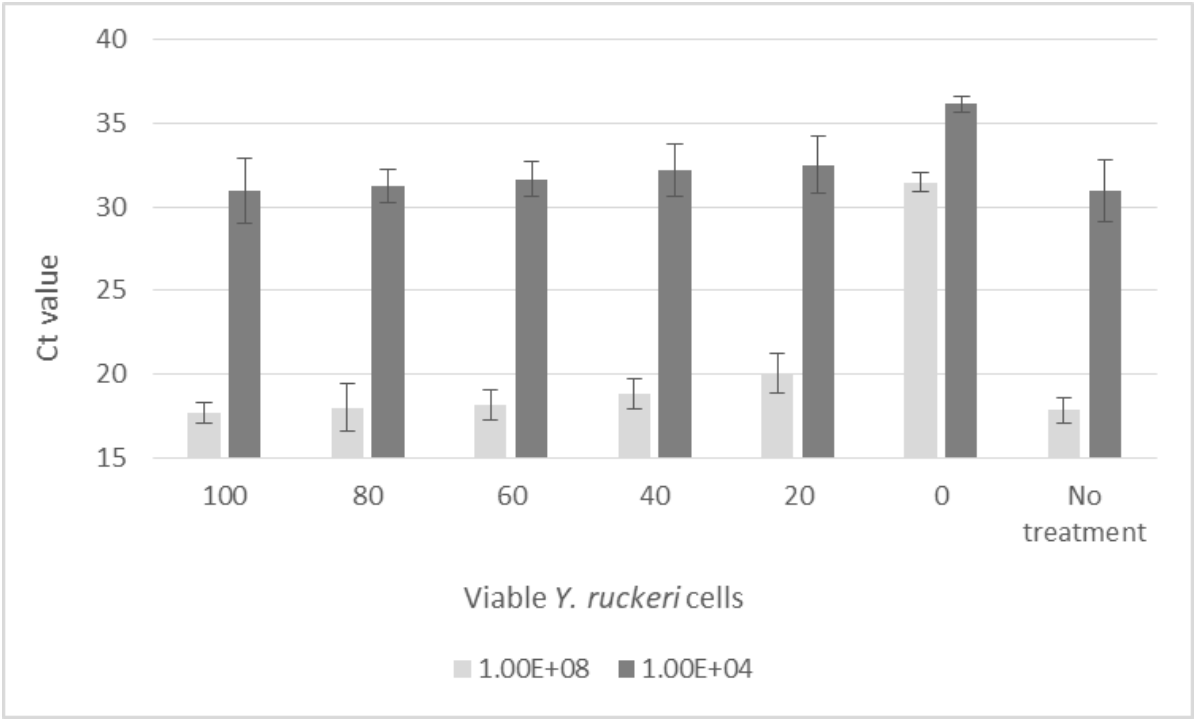

Figure 40. PEMAX v-qPCR results for ratios of Live:Dead *Y. ruckeri* cells spiked in kidney tissue (10^4 and 10^8 CFU mL^{-1}). *n* = 3.

At 10^8 CFU mL^{-1} in a matrix of kidney tissue, PEMAX v-qPCR results for 0 % live cells were higher than the sample containing 100 % live cells by an average Ct value of 12.7. The Ct value of samples gradually increased as the percentage of dead cells increased (Figure 40). The correlation coefficient was -0.75.

When only treated dead cells were present in the sample, conventional vPCR resulted in a very weak amplicon in all replicates, however complete supression was not seen (Table 15).

Table 15: Conventional PEMAX vPCR on live and dead treated *Y. ruckeri* cells in spiked kidney tissue. The number in the results column equals the number of replicates out of three that produced an amplicon. The red numbers indicate all replicates produced a very weak amplicon when compared to the live samples.

Final concentration of bacteria (CFU mL^{-1})	Percent live cells	Result
10^8	100	3
	80	3
	60	3
	40	3
	20	3
	0	3
	100 (no treatment)	3
10^4	100	3
	80	3
	60	3
	40	3
	20	3
	0	3
	100 (no treatment)	3

Tenacibaculum maritimum

At 10^3 CFU mL^{-1} in a matrix of skin tissue, PEMAX v-qPCR results for 0 % live cells (100 % dead) were higher than those 100 % live cells by an average Ct value of 7.83. The Ct values of samples gradually increased as the percentage of dead cells increased (Figure 41). The correlation co-efficient was -0.88.

At 10^6 CFU mL^{-1} in a matrix of skin tissue, PEMAX v-qPCR results for 0 % live cells were higher than those 100 % live cells by an average Ct value of 10.57. The Ct values of samples increased as the percentage of dead cells increased (Figure 41). The correlation co-efficient was -0.88.

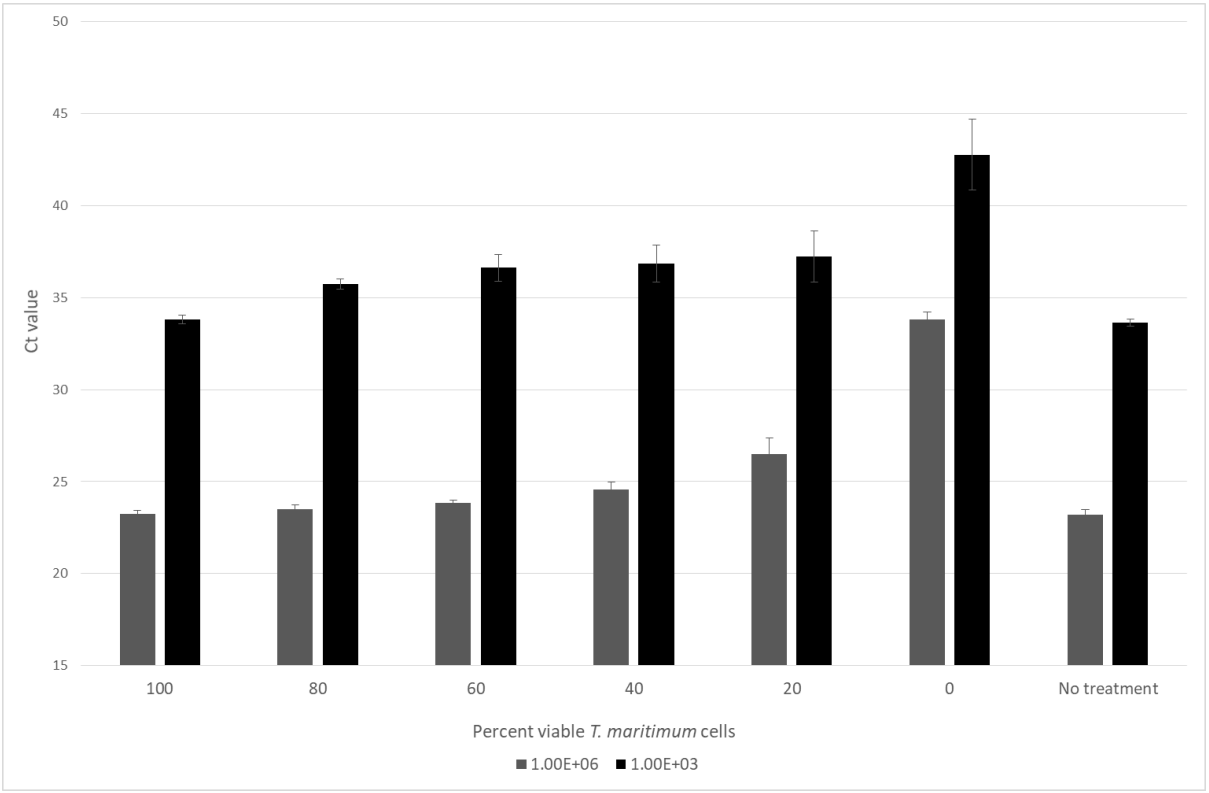

Figure 41. Bars represent average Ct value of ratios of Live: Dead *T. maritimum* live and dead cells spiked in skin tissue (10^3 and 10^6 CFU mL^{-1}) exposed to PEMAX. *n* = 3. Error bars represent SD between replicates.

At a final concentration of 10^3 CFU mL^{-1}, the number of conventional PCR replicates that produced an amplicon when live cells were present was lower when there were 60 % or less live cells. This result indicates that this cell concentration is at the LOD of the assay. No amplicons were produced when there were only dead cells in the sample.

At a final concentration of 10^6 CFU mL^{-1}, complete suppression of PEMAX treated samples via conventional PCR was observed when only dead cells were present. This indicates that complete suppression is achieved in this assay at this concentration of bacteria (10^6 CFU mL^{-1}) and below. All samples produced an amplicon when there was a mix of live and dead cells (Table 16).

Table 16: Conventional PEMAX vPCR on live and dead *T. maritimum* cells in spiked skin tissue. The number in the results column equals the number of replicates out of three that produced an amplicon.

Final concentration of bacteria (CFU mL^{-1})	Percent live cells	Result
10^6	100	3
	80	3
	60	3
	40	3
	20	3
	0	0
	100 (no treatment)	3
10^3	100	3
	80	3
	60	2
	40	2
	20	1
	0	0
	100 (no treatment)	3

ABV

As RNA from dead viral cells degrades rapidly in tissue it was unsurprising that there was often no amplification in the conventional RT-PCR whether the sample was treated with dye or not. During this experiment it was found that the virus stocks used had lost viability after being stored at -80 °C for 3 months (reduced by 4 logs of concentration, data not shown). Upon discovery, only fresh stocks or stocks stored for < 1 month were used for experiments.

Using neat virus (final concentration, 10^4 $TCID_{50}$), all samples amplified in the conventional RT-PCR when there was live virus present (Table 17). Samples with a final concentration of 10^2 $TCID_{50}$ did not produce an amplicon as this concentration is below the LOD of the assay.

Table 17: Results of conventional RT-PCR for ABV when spiked in liver tissue at different concentrations of live and dead virus. The numbers in the results columns equals the number of replicates out of three that produced an amplicon.

Final concentration of virus ($TCID_{50}$)	Percent live cells	Result
10^4	100	3
	80	3
	60	3
	40	3
	20	3
	0	0
	100 (no treatment)	3
10^2	100	0
	80	0
	60	0
	40	0
	20	0
	0	0
	100 (no treatment)	0

4.15 PROTOCOL FOR REPEATABILITY ON ARTIFICIALLY SPIKED TISSUE

Yersinia ruckeri

At 10^7 CFU mL^{-1} in a matrix of kidney tissue, v-qPCR results for 0 % live cells that were treated with PEMAX resulted in an average Ct value of 8.73 higher than the equivalent untreated samples. PEMAX treated samples that contained 50 % live cells had Ct values that were, on average, 2 Ct values lower than the equivalent untreated samples (Figure 42).

At 10^3 CFU mL^{-1} in a matrix of kidney tissue, v-qPCR results for 0 % live cells that were treated with PEMAX revealed an average Ct value of 3.5 higher than the equivalent untreated samples. Five of the nine samples gave no signal in the v-qPCR, i.e., complete suppression.

PEMAX treated samples that contained 50 % live cells had Ct values that were, on average, 2.43 higher than the equivalent untreated samples. One of the nine samples gave no signal in the v-qPCR assay. PEMAX treated samples that contained only live cells had Ct values that were, on average, 3.14 higher than untreated samples. All samples in this group amplified in the v-qPCR.

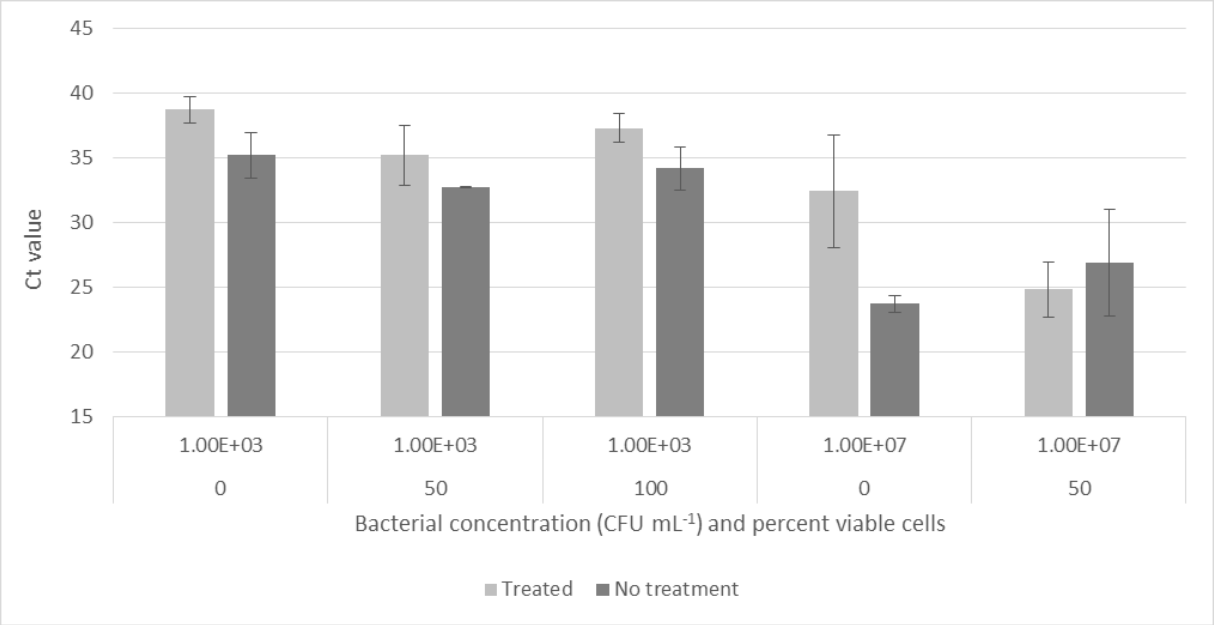

Figure 42. Bars represent average Ct values of PEMAX treated and untreated samples with varying amounts of live *Y. ruckeri* cells at two bacterial concentrations (10^7 and 10^3 CFU mL^{-1}). *n* = 3. Error bars represent SD between replicates.

These results show that above the limit of detection (> 10^4 CFU mL^{-1}), a clear differentiation exists between samples containing live or dead cells. In a sample containing an even ratio of mixed cells, the difference in Ct values was small indicating the presence of live cells.

Tenacibaculum maritimum

At 10^5 CFU mL^{-1} in a matrix of skin tissue, v-qPCR results for samples that contained 0 % live cells and were treated resulted in Ct values that were on average 8.76 higher than the equivalent untreated samples. PEMAX treated samples that contained 50 % live cells had Ct values on average 2.39 Ct higher than the equivalent untreated samples (Figure 43).

At 10^3 CFU mL^{-1} in a matrix of skin tissue, v-qPCR results for 0 % live cells that were treated with PEMAX were higher than the equivalent untreated samples by an average Ct value of 6.25. Eight of these nine samples gave no signal in the v-qPCR, i.e., complete suppression. PEMAX treated samples that contained 50 % live cells had Ct values on average, 2.36 higher than the equivalent untreated samples. PEMAX treated samples that contained only live cells had Ct values that were on average 1.23 higher than the equivalent untreated samples.

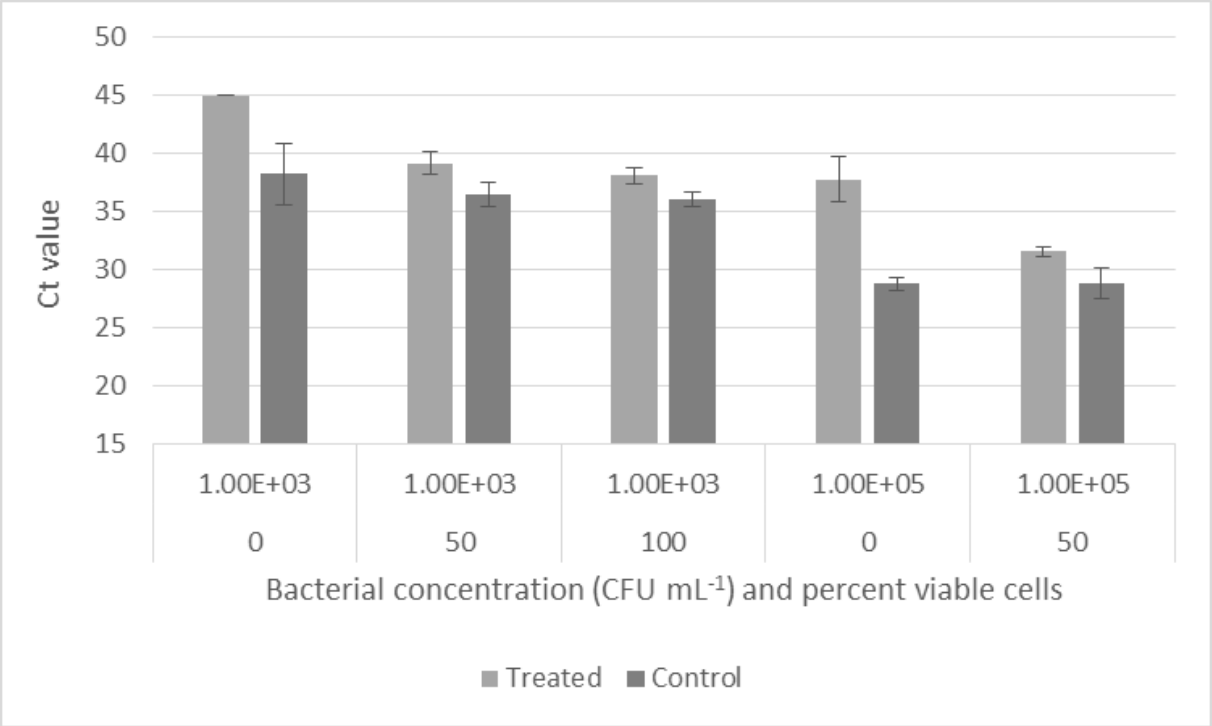

Figure 43. Bars represent the average Ct values of PEMAX treated and untreated samples with varying amounts of live *T. maritimum* cells at two bacterial concentrations (10^5 and 10^3 CFU mL^{-1}). *n* = 3. Error bars represent the SD between replicates.

Conventional vPCR performed as expected for samples with a final bacterial concentration of 10^5 CFU mL^{-1} whereby samples containing dead cells that were treated with PEMAX did not produce an amplicon and samples containing PEMAX treated live cells produced an amplicon. Conventional vPCR on samples with a final concentration of 10^3 CFU mL^{-1} produced variable results when the sample contained live cells but produced expected results when samples contained only dead cells (Table 18). These results were not unexpected as this is at the LOD of the assay and the Ct values indicate that it is at the limit of result reliability (i.e., > 36).

Table 18: Results of conventional vPCR when spiked in skin tissue at different concentrations of live and dead *T. maritimum*. The numbers in the results columns equals the number of replicates that produced an amplicon. Red = untreated with dye.

Final bacterial concentration (CFU mL^{-1})	Dye treated	Sample	Result
10^3	Yes	Dead 100 %	0/9
	No	Dead 100 %	3/3
	Yes	Live 50 %	4/9
	No	Live 50 %	3/3
	Yes	Live 100 %	4/9
	No	Live 100 %	3/3
10^5	Yes	Dead 100 %	0/9
	No	Dead 100 %	3/3
	Yes	Live 50 %	9/9
	No	Live 50 %	3/3

ABV

No amplification in the conventional RT-PCR was observed for PEMAX treated dead cells in a matrix of liver tissue spiked to a final concentration of 10^4 $TCID_{50}$. Untreated spiked samples (10^4 $TCID_{50}$) also showed no amplification in the conventional RT-PCR.

The conventional RT-PCR produced amplification in both PEMAX treated and untreated samples that contained 50 % live cells. One replicate that was treated with dye did not amplify. Similarly, the conventional RT-PCR produced amplification in both PEMAX treated and untreated samples of 100 % live cells in a matrix of liver tissue.

These results showed that there was no effect of the PEMAX dye on live cells. Although this was not measured, a reduced intensity of the amplification was observed in samples which contained 50 % live cells. No amplification was seen in samples containing PEMAX treated dead cells (100 %). However, no amplification was observed in untreated samples that contained dead virus.

The conventional RT-PCR carried out on PEMAX treated dead cells in a matrix of liver tissue spiked with a final concentration of 10^3 $TCID_{50}$ and samples that contained PEMAX treated 50 % live cells produced no amplicon. Similarly, the conventional RT-PCR produced no amplicon for untreated 50 % live samples.

These results show that the dead viral RNA in the tissue sample is degrading within 30 min. Similarly, Seear & Sweeny (2008) reported a reduction in RNA integrity in the liver tissues of salmon after 1 h post-mortem. However, the process of heating the virus prior to adding to the liver tissue is also expected to influence this degradation and also serves as a likely explanation of these results. As 10^3 $TCID_{50}$ is the LOD of the assay, these results are not unexpected (Table 19).

To fully understand the applicability of this assay for ABV, naturally infected tissues would need to be examined to see if a dead virus in a sample acted that same way as observed in artificially spiked tissues. While the technology looks promising for this pathogen, further validation work is required before use in a regulatory setting.

Table 19: Results of conventional RT-PCR when spiked in liver tissue at different concentrations of live and dead Aquabirnavirus. The numbers in the results columns equals the number of replicates that produced an amplicon. Red = not treated with dye.

Final viral concentration ($TCID_{50}$)	Dye treated	Sample	Result
10^4	Yes	Dead 100 %	0/9
	No	Dead 100 %	0/9
	Yes	Live 50 %	8/9
	No	Live 50 %	3/3
	Yes	Live 100 %	9/9
	No	Live 100 %	3/3
10^3	Yes	Dead 100 %	0/9
	No	Dead 100 %	0/3
	Yes	Live 50 %	0/9
	No	Live 50 %	0/3

4.16 PROFICIENCY TESTING

Yersinia ruckeri

Two users at the same laboratory: There were no significant differences observed between the v-qPCR results produced by two users at the same laboratory (AHL) (Table 20).

Two users, different laboratory: The panel sent to PHEL took two days to arrive. The PEMAX v-qPCR results produced from this panel were unexpected and included amplification in sample 20, a sample that was not spiked with *Y. ruckeri* (Appendix 4).

To determine if the differences observed were due to the transit time or due to the different user and setting, panels were set up in triplicate and processed in the following ways:

- One set was processed at AHL immediately after preparation.
- One set was sent to PHEL for processing on arrival.
- One set was processed at AHL at the same time as PHEL.

These samples took one day to arrive to PHEL. No significant differences were observed in any of the PEMAX v-qPCR results produced (Table 21).

Note, sample 20 for both trials was kidney tissue that was not spiked with *Y. ruckeri*. This sample gave no signal in the qPCR in any panel. For other samples where no signal was obtained, a Ct value of 45 was ascribed which was the highest cycle number the assay runs to.

Table 20: v-qPCR Ct values from the panel of samples spiked with *Y. ruckeri* run in parallel with two users within AHL. Red = treated with PEMAX.

Sample	User 1 (Ct value)	User 2 (Ct value)	Average (Ct value)	SD
10^8 L	19.02	17.85	18.44	0.83
10^8 L	20.65	17.87	19.26	1.96
10^8 D	26.37	27.51	26.94	0.81
10^8 D	19.96	19.79	19.87	0.12
10^7 L 10^5 D	23.38	21.75	22.56	1.16
10^7 L 10^5 D	22.32	22.16	22.24	0.11
10^6 D	35.53	32.82	34.18	1.92
10^6 D	28.54	29.03	28.78	0.35
10^6 L	25.25	29.62	27.44	3.09
10^6 L	24.64	27.46	26.05	1.99
10^5 L	29.70	30.79	30.25	0.77
10^5 L	28.82	31.78	30.30	2.10
10^4 D	40.00	35.84	37.92	2.94
10^4 D	35.64	32.38	34.01	2.30
10^4 L	31.66	31.05	31.35	0.43
10^4 L	31.31	31.58	31.45	0.19
10^8 L 10^8 D	20.00	18.46	19.23	1.09
10^8 L 10^8 D	19.91	18.75	19.33	0.82
10^6 D	37.80	31.68	34.74	4.33
10^6 D	28.12	26.76	27.44	0.96
10^6 L 10^4 D	25.85	24.93	25.39	0.65
10^6 L 10^4 D	24.96	25.88	25.42	0.65
10^7 D	33.69	23.65	28.67	7.10
10^7 D	24.17	21.60	22.89	1.82
10^7 L	21.53	22.59	22.06	0.75
10^7 L	21.78	21.38	21.58	0.28
10^8 D	27.93	24.06	25.99	2.74
10^8 D	21.24	18.60	19.92	1.87
10^4 D	40.00	36.67	38.33	2.36
10^4 D	34.16	33.48	33.82	0.48
10^4 L	32.18	30.95	31.56	0.87
10^4 L	31.51	31.16	31.34	0.25
10^5 L 10^5 D	29.77	29.26	29.51	0.36
10^5 L 10^5 D	29.13	28.77	28.95	0.25

Table 21: v-qPCR Ct values from the panel of samples spiked with *Y. ruckeri* run in parallel with two users at the different laboratories; AHL and PHEL. Red = treated with PEMAX.

Sample	AHL (Ct value)	PHEL (Ct value)	Average (Ct value)	SD
10^8 L	18.65	18.18	18.42	0.33
10^8 L	17.07	17.43	17.25	0.25
10^8 D	28.87	26.83	27.85	1.44
10^8 D	21.67	21.44	21.55	0.17
10^7 L 10^5 D	20.62	20.45	20.54	0.12
10^7 L 10^5 D	19.35	21.36	20.35	1.42
10^6 D	31.10	30.30	30.70	0.56
10^6 D	27.78	26.47	27.12	0.92
10^6 L	21.44	22.27	21.85	0.58
10^6 L	20.73	22.70	21.71	1.39
10^5 L	26.19	23.95	25.07	1.58
10^5 L	25.88	23.87	24.88	1.42
10^5 D	36.38	36.11	36.24	0.19
10^5 D	31.38	31.87	31.62	0.35
10^5 L 10^5 D	25.55	23.10	24.32	1.74
10^5 L 10^5 D	24.94	24.10	24.51	0.60
10^4 D	38.05	36.72	37.38	0.94
10^4 D	32.38	34.90	33.64	1.78
10^4 L	29.43	28.71	29.07	0.51
10^4 L	28.38	29.65	29.02	0.90
10^8 L 10^8 D	19.24	18.37	18.80	0.62
10^8 L 10^8 D	17.77	18.42	18.10	0.46
10^6 D	32.43	24.1	28.27	5.89
10^6 D	27.63	29.54	28.59	1.35
10^6 L 10^4 D	21.82	21.34	21.58	0.34
10^6 L 10^4 D	21.91	22.33	22.12	0.30
10^7 D	30.61	29.61	30.11	0.71
10^7 D	24.70	24.69	24.69	0.01
10^7 L	19.07	17.45	18.26	1.15
10^7 L	18.44	17.5	17.97	0.66
10^8 D	25.72	25.01	25.37	0.50
10^8 D	21.29	22.19	21.74	0.64
10^4 D	45.00	38.00	41.50	4.95
10^4 D	33.07	32.05	32.56	0.72
10^4 L	30.28	28.73	29.51	1.10
10^4 L	28.19	27.68	27.94	0.36
10^5 L 10^5 D	25.42	24.75	25.08	0.47
10^5 L 10^5 D	23.13	22.76	22.95	0.26

Tenacibaculum maritimum

Two users, same laboratory:

No significant differences were observed in the v-qPCR results between the two users (Table 22). Sample 20 of the panel was skin tissue that was not spiked with *T. maritimum* and gave no signal in the qPCR in any panel.

Two samples did not give consistent results between the two users in the conventional vPCR assay. These samples had the lowest bacterial concentration (10^2 CFU mL^{-1}) and were below the LOD of the assay. This discrepancy was not unexpected and is most likely due to the random amplification of such a low concentration of bacteria.

Table 22: v-qPCR and vPCR results from from the panel of samples spiked with *T. maritimum* run by two users in the same laboratory. Y = Yes, N = No. Red = treated with dye. * samples that were not consistent between users in the conventional vPCR.

Sample	User 1 (Ct value)	User 2 (Ct value)	Average (Ct value)	SD	Conventional amplicon user 1	Conventional amplicon user 2
10^6 L	27.81	28.18	28.00	0.26	Y	Y
10^6 L	26.58	27.26	26.92	0.48	Y	Y
10^6 D	36.46	36.78	36.62	0.23	N	N
10^6 D	26.94	26.62	26.78	0.23	Y	Y
10^5 L 10^3 D	31.94	32.29	32.12	0.25	Y	Y
10^5 L 10^3 D	31.55	31.61	31.58	0.04	Y	Y
10^4 D	39.07	40.75	39.91	1.19	N	N
10^4 D	36.23	38.17	37.20	1.38	Y	Y
10^4 L	33.00	33.40	33.20	0.29	Y	Y
10^4 L	33.40	32.54	32.97	0.61	Y	Y
10^3 L	38.58	38.88	38.73	0.22	N	N
10^3 L	36.02	36.18	36.10	0.12	Y	Y
10^3 D	39.98	38.63	39.31	0.96	N	N
10^3 D	35.91	35.67	35.79	0.17	Y	Y
10^3 L 10^3 D	37.92	38.36	38.14	0.31	Y	Y
10^3 L 10^3 D	36.50	37.51	37.00	0.71	Y	Y
10^2 D	Neg	Neg	Neg	-	N	N
10^2 D	41.57	43.85	42.71	1.61	Y	Y
10^2 L*	42.59	41.65	42.12	0.66	Y	N
10^2 L	38.86	38.80	38.83	0.04	Y	Y
10^6 L 10^6 D	27.39	28.32	27.86	0.66	Y	Y
10^6 L 10^6 D	26.21	26.71	26.46	0.36	Y	Y
10^4 D	36.15	37.55	36.85	0.99	N	N
10^4 D	34.37	35.37	34.87	0.71	Y	Y
10^4 L 10^2 D	34.94	35.35	35.14	0.29	Y	Y
10^4 L 10^2 D	33.29	33.53	33.41	0.17	Y	Y
10^5 D	39.95	42.78	41.36	2.00	N	N
10^5 D	30.13	30.92	30.52	0.56	Y	Y
10^5 L	30.68	31.03	30.86	0.25	Y	Y
10^5 L	29.92	29.79	29.86	0.09	Y	Y
10^6 D	28.24	29.32	28.78	0.76	N	N
10^6 D	26.60	27.70	27.15	0.78	Y	Y
10^2 D	Neg	Neg	Neg	-	N	N
10^2 D*	39.71	41.97	40.84	1.59	Y	N
10^2 L	41.97	41.33	41.65	0.45	N	N
10^2 L	40.37	39.15	39.76	0.86	Y	Y
10^5 L 10^5 D	34.82	34.99	34.90	0.12	Y	Y
10^5 L 10^5 D	33.47	33.22	33.35	0.18	Y	Y

ABV

Two users, same laboratory:

The amplicons produced in the RT-PCR showed there was no difference between the two users (Table 23).

No difference was observed in treated live cells compared untreated live cells. All results were consistent with previous experiments where dead virus did not produce an amplicon on the conventional RT-PCR regardless of dye treatment.

Dead virus was extracted alongside the samples and not incorporated into the liver homogenates. These samples revealed only the virus at a neat concentration produced an amplicon and further dilutions showed no amplification. The concentration of virus in this experiment was the same as the dilutions and explains why no amplification was seen in these spiked samples.

For live cells not spiked into tissue or dye treated the following concentrations amplified: 7×10^4, 7×10^3 and 7×10^2 $TCID_{50}$. This correlated with the results seen in the liver samples spiked with virus at these dilutions.

Table 23: Conventional RT-PCR results from a panel of 20 tissue samples spiked with ABV at varying concentrations run by two users in the same laboratory. Red = treated with dye.

Sample ($TCID_{50}$)	User 1	User 2
7×10^3 L	Positive	Positive
7×10^3 L	Positive	Positive
7×10^3 D	Negative	Negative
7×10^3 D	Negative	Negative
7×10^3 L, 7×10^3 D	Positive	Positive
7×10^3 L, 7×10^3 D	Positive	Positive
7×10^2 L, 7×10^3 D	Positive	Positive
7×10^2 L, 7×10^3 D	Positive	Positive
7×10^2 D	Negative	Negative
7×10^2 D	Negative	Negative
7×10^3 L, 7×10^2 D	Positive	Positive
7×10^3 L, 7×10^2 D	Positive	Positive
7×10^2 L	Positive	Positive
7×10^2 L	Positive	Positive
7×10^2 D	Positive	Positive
7×10^2 D	Positive	Positive
-	Negative	Negative
-	Negative	Negative
7×10^2 D	Negative	Negative
7×10^2 D	Negative	Negative
7×10^3 L, 7×10^2 D	Positive	Positive
7×10^3 L, 7×10^2 D	Positive	Positive
7×10^2 L, 7×10^3 D	Positive	Positive
7×10^2 L, 7×10^3 D	Positive	Positive
7×10^2 D	Negative	Negative
7×10^2 D	Negative	Negative
7×10^2 L	Positive	Positive
7×10^2 L	Positive	Positive
7×10^2 L, 7×10^2 D	Positive	Positive
7×10^2 L, 7×10^2 D	Positive	Positive
7×10^2 L, 7×10^3 D	Negative	Negative
7×10^2 L, 7×10^3 D	Negative	Negative
7×10^3 L	Positive	Positive
7×10^3 L	Positive	Positive
7×10^3 D	Negative	Negative
7×10^3 D	Negative	Negative
7×10^2 D	Negative	Negative
7×10^2 D	Negative	Negative
7×10^1 L	Negative	Negative
7×10^1 L	Negative	Negative

4.17 DETERMINING THE CUT-OFF POINT FOR QPCR

Yersinia ruckeri

Ct values were compiled from all experiments carried out using the optimal protocol. This gave 85 data points from which the % difference between the PEMAX treated and untreated samples was determined (Table 24).

Table 24: % Δ Ct (and range) between PEMAX treated and untreated samples containing live cells, dead cells, or a mix of cells.

Sample type	% Δ Ct†
Live (*n* = 49)	0.28 (0 – 2.81)
Dead (36)*	5.55 (0.83 – 20.79)

† A significant difference was seen between the live and dead samples ($p < 0.0001$).
* Four cases showed a Ct value in the dye untreated samples but were not detected in the dye treated samples.

An indicative value of 1 % cut-off in % difference between PEMAX treated and untreated samples was ascribed. That is, a value of ≤ 1 % was likely to have live cells and anything > 1 % was likely to have only dead cells. This was consistent for bacterial concentrations > 10^3 CFU mL^{-1} (98 % accurate, Table 25). However, results were not reliable at ≤ 10^3 CFU mL^{-1} (87 % accurate). This aligns with the LOD of the assay where anything ≥ a Ct of 32 will not provide consistent results.

Table 25: Summary of all data compiled from experiments where optimal protocol was used on live and dead *Y. ruckeri* cells. Red = data that does not conform to the cut-off value. Blue = samples that had no signal in the qPCR (given Ct 40 for analysis). L = Live; D = Dead.

Live cell concentration (log_{10})	Dead cell concentration (log_{10})	No treatment (Ct value)	Treatment (Ct value)	Treated – not treated (Ct value)	Change in Ct value not treated (%)	Change in Ct value treated (%)	Difference treated – not treated (%)	State of cells based on a 1 % cut-off value
0	7	24.11	32.59	8.48	35.17	26.02	9.15	D
0	7	24.11	29.32	5.21	21.61	17.77	3.84	D
0	7	24.11	29.13	5.02	20.82	17.23	3.59	D
0	7	22.90	27.67	4.77	20.83	17.24	3.59	D
0	7	22.90	28.59	5.69	24.85	19.90	4.95	D
0	7	22.90	32.16	9.26	40.44	28.79	11.64	D
0	7	24.00	37.72	13.72	57.17	36.37	20.79	D
0	7	24.00	37.09	13.09	54.54	35.29	19.25	D
0	7	19.79	27.51	7.72	39.01	28.06	10.95	D
0	7	18.60	24.06	5.46	29.35	22.69	6.66	D
0	7	20.52	25.39	4.88	23.77	19.21	4.57	D
0	7	21.14	28.81	7.67	36.26	26.61	9.65	D
0	7	20.78	28.34	7.56	36.39	26.68	9.71	D
0	7	20.68	25.68	5.00	24.18	19.47	4.71	D
7	7	18.75	18.46	-0.29	-1.55	-1.57	0.02	L
7	7	20.43	20.21	-0.22	-1.06	-1.07	0.01	L

Live cell concentration (log_{10})	Dead cell concentration (log_{10})	No treatment (Ct value)	Treatment (Ct value)	Treated – not treated (Ct value)	Change in Ct value not treated (%)	Change in Ct value treated (%)	Difference treated – not treated (%)	State of cells based on a 1 % cut-off value
7	7	19.43	19.50	0.07	0.37	0.37	0.00	L
6	6	27.22	23.50	-3.72	-13.67	-15.83	2.16	D
6	6	27.22	25.50	-1.72	-6.32	-6.75	0.43	L
6	6	27.22	28.74	1.52	5.58	5.29	0.30	L
6	6	30.80	29.78	-1.02	-3.31	-3.43	0.11	L
6	6	22.56	22.24	-0.32	-1.42	-1.44	0.02	L
6	6	22.56	21.83	-0.73	-3.24	-3.34	0.11	L
6	6	22.56	22.40	-0.16	-0.71	-0.71	0.01	L
0	6	22.47	26.04	3.57	15.89	13.71	2.18	D
0	6	24.83	34.15	9.32	37.56	27.30	10.26	D
0	6	23.75	32.86	9.11	38.36	27.73	10.64	D
6	6	22.33	22.08	-0.25	-1.11	-1.12	0.01	L
6	6	21.30	21.23	-0.07	-0.31	-0.31	0.00	L
0	5	26.76	31.68	4.92	18.39	15.53	2.86	D
0	5	30.15	37.57	7.42	24.61	19.75	4.86	D
0	5	27.39	36.93	9.54	34.83	25.83	9.00	D
0	5	28.62	33.93	5.31	18.56	15.65	2.91	D
0	5	27.93	36.80	8.87	31.78	24.12	7.66	D
6	5	22.16	21.75	-0.41	-1.85	-1.89	0.03	L
0	5	29.03	32.82	3.79	13.06	11.55	1.51	D
0	4	31.82	38.55	6.73	21.17	17.47	3.70	D
6	4	22.97	24.01	1.05	4.56	4.36	0.20	L
6	4	21.95	22.62	0.68	3.09	3.00	0.09	L
4	4	28.77	29.26	0.49	1.70	1.67	0.03	L
4	4	29.59	29.37	-0.22	-0.74	-0.75	0.01	L
4	4	29.44	29.54	0.10	0.35	0.35	0.00	L
4	4	28.41	28.46	0.04	0.14	0.14	0.00	L
4	4	28.41	29.32	0.92	3.22	3.12	0.10	L
0	3	33.15	37.07	3.92	11.82	10.57	1.25	D
0	3	32.43	37.90	5.47	16.87	14.43	2.43	D
0	3	34.64	40.00	5.36	15.49	13.41	2.08	D
0	3	33.47	40.00	6.53	19.52	16.33	3.19	D
0	3	34.80	40.00	5.20	14.93	12.99	1.94	D
0	3	34.02	40.00	5.98	17.57	14.94	2.62	D
5	3	25.88	24.93	-0.95	-3.67	-3.81	0.14	L
5	3	24.81	26.08	1.28	5.15	4.90	0.25	L
5	3	24.54	25.60	1.05	4.29	4.11	0.18	L
3	3	31.64	31.11	-0.53	-1.68	-1.70	0.03	L

Live cell concentration (log_{10})	Dead cell concentration (log_{10})	No treatment (Ct value)	Treatment (Ct value)	Treated – not treated (Ct value)	Change in Ct value not treated (%)	Change in Ct value treated (%)	Difference treated – not treated (%)	State of cells based on a 1 % cut-off value
3	3	32.27	32.48	0.22	0.67	0.67	0.00	L
3	3	30.73	31.26	0.54	1.75	1.72	0.03	L
0	3	37.01	32.95	-4.06	-10.97	-12.32	1.35	D
0	3	33.48	37.72	4.24	12.66	11.24	1.42	D
0	3	33.48	38.31	4.83	14.43	12.61	1.82	D
0	3	35.05	39.18	4.13	11.78	10.54	1.24	D
0	3	32.38	35.84	3.46	10.69	9.65	1.03	L
0	3	33.48	36.67	3.19	9.53	8.70	0.83	L
2	2	32.73	33.09	0.36	1.10	1.09	0.01	L
2	2	32.73	32.62	-0.11	-0.34	-0.34	0.00	L
2	2	32.73	33.07	0.34	1.04	1.03	0.01	L
3	0	32.97	38.84	5.87	17.80	15.11	2.69	D
3	0	32.97	38.98	6.01	18.23	15.42	2.81	D
7	0	17.85	17.87	0.02	0.11	0.11	0.00	L
7	0	21.04	19.41	-1.62	-7.72	-8.36	0.65	L
7	0	19.88	19.86	-0.02	-0.09	-0.09	0.00	L
6	0	20.51	20.21	-0.30	-1.46	-1.48	0.02	L
5	0	27.46	29.62	2.16	7.87	7.29	0.57	L
5	0	25.17	26.35	1.19	4.71	4.50	0.21	L
5	0	24.07	25.63	1.57	6.50	6.11	0.40	L
4	0	31.78	30.79	-0.99	-3.12	-3.22	0.10	L
4	0	29.45	28.60	-0.85	-2.90	-2.99	0.09	L
4	0	28.40	29.43	1.04	3.65	3.52	0.13	L
3	0	32.97	34.15	1.18	3.58	3.46	0.12	L
3	0	35.29	35.34	0.05	0.14	0.14	0.00	L
3	0	35.29	39.27	3.98	11.28	10.13	1.14	L
3	0	35.29	33.40	-1.89	-5.36	-5.66	0.30	L
3	0	31.58	31.05	-0.53	-1.68	-1.71	0.03	L
3	0	31.16	30.95	-0.21	-0.67	-0.68	0.00	L
3	0	31.13	31.14	0.01	0.04	0.04	0.00	L
3	0	30.63	31.65	1.03	3.35	3.24	0.11	L

Tenacibaculum maritimum

Ct values were compiled from all experiments carried out using the optimal protocol. This gave 108 data points from which the % difference between the PEMAX treated and untreated samples was determined (Tables 26 and 27).

Table 26: % Δ Ct (and range) between PEMAX treated and PEMAX untreated samples containing live cells, dead cells, or a mix of cells.

Sample type	% Δ Ct†
Live (*n* = 76)	0.37 (0 – 2.51)
Dead (27)*	5.09 (0.36 – 10.64)

† A significant difference was seen between the live and dead samples ($p < 0.0001$).
* Six cases showed a Ct value in the PEMAX untreated samples but were not detected in the PEMAX treated samples.

A 2.5 % cut-off in % difference between PEMAX treated and untreated samples was ascribed. That is, values ≤ 2.5 % were likely to have live cells and anything > 2.5 % was likely to have only dead cells. This was consistent for bacterial concentrations > 10^3 CFU mL^{-1} (98 % accurate, Table 26). However, results were not reliable ≤ 10^3 CFU mL^{-1} (88 % accurate) which aligns with the results expected for the LOD of the assay.

For interpretation, if there is a late Ct (e.g., 38) in the non-treated sample and no Ct value in the treated sample there is likely only dead cells in the sample. A value of 45 was used for analysis which meant the cut-off value was exceeded (i.e., the % difference was < 2.5 %), however they are still interpreted as dead as there was no signal. An enrichment protocol may help clarify these late Ct values.

Table 27: Summary of all data compiled from experiments where optimal protocol was used on live and dead *T. maritimum* cells. Red = data that does not conform to the ascribed cut-off value. Blue text = samples that had no signal in the qPCR but were given 45 for analysis. Blue background = samples that had no signal once they had been treated but the cut-off was less than 2.5 %. L = Live; D = Dead.

Live cell concentration (log_{10})	Dead cell concentration (log_{10})	No treatment (Ct value)	Treatment (Ct value)	Treated – not treated (Ct value)	Change in Ct value not treated (%)	Change in Ct value treated (%)	Difference treated – not treated (%)	State of cells based on a 2.5 % cut-off value
6	6	26.71	28.32	1.61	6.03	5.69	0.34	L
6	6	26.21	27.39	1.18	4.51	4.31	0.19	L
6	6	26.20	26.00	-0.21	-0.79	-0.79	0.01	L
6	6	25.95	26.35	0.40	1.53	1.51	0.02	L
6	6	24.61	25.50	0.89	3.62	3.49	0.13	L
6	6	25.30	28.17	2.87	11.34	10.18	1.15	L
5	6	28.44	30.53	2.09	7.36	6.86	0.50	L
5	6	28.36	30.52	2.16	7.61	7.07	0.54	L
5	6	25.45	27.29	1.84	7.22	6.73	0.49	L
4	6	31.07	33.53	2.46	7.93	7.35	0.58	L
4	6	31.01	33.44	2.44	7.86	7.29	0.57	L
0	6	26.62	36.78	10.17	38.19	27.64	10.56	D
0	6	26.94	36.46	9.52	35.35	26.12	9.23	D
0	6	27.45	37.11	9.66	35.18	26.02	9.16	D
0	6	26.30	35.85	9.55	36.31	26.64	9.67	D
5	5	33.22	34.99	1.76	5.31	5.04	0.27	L
5	5	33.47	34.82	1.35	4.04	3.88	0.16	L
0	5	29.35	38.98	9.63	32.81	24.70	8.11	D
0	5	28.31	37.54	9.23	32.60	24.59	8.02	D

Live cell concentration (log_{10})	Dead cell concentration (log_{10})	No treatment (Ct value)	Treatment (Ct value)	Treated – not treated (Ct value)	Change in Ct value not treated (%)	Change in Ct value treated (%)	Difference treated – not treated (%)	State of cells based on a 2.5 % cut-off value
0	5	29.35	38.98	9.63	32.81	24.70	8.11	D
0	5	30.92	42.78	11.86	38.36	27.73	10.64	D
0	5	30.13	39.95	9.82	32.58	24.57	8.01	D
0	5	30.46	41.13	10.67	35.01	25.93	9.08	D
4	4	30.46	30.89	0.43	1.42	1.40	0.02	L
4	4	28.15	30.86	2.71	9.63	8.78	0.85	L
4	4	30.46	30.89	0.43	1.42	1.40	0.02	L
0	4	34.41	43.75	9.33	27.12	21.33	5.79	D
0	4	30.64	39.80	9.16	29.88	23.01	6.87	D
0	4	30.64	39.80	9.16	29.88	23.01	6.87	D
0	4	33.69	40.60	6.91	20.50	17.01	3.49	D
5	3	31.61	32.29	0.68	2.16	2.12	0.05	L
5	3	31.55	31.94	0.39	1.24	1.22	0.02	L
5	3	27.48	28.08	0.60	2.18	2.14	0.05	L
5	3	27.48	28.08	0.60	2.18	2.14	0.05	L
5	3	29.43	33.81	4.38	14.88	12.96	1.93	L
3	3	37.51	38.36	0.85	2.27	2.22	0.05	L
3	3	36.50	37.92	1.42	3.88	3.73	0.14	L
3	3	36.76	35.87	-0.89	-2.42	-2.48	0.06	L
3	3	38.35	39.36	1.01	2.63	2.56	0.07	L
0	3	38.17	40.75	2.57	6.75	6.32	0.43	L
0	3	35.37	37.55	2.17	6.15	5.79	0.36	L
0	3	36.23	39.07	2.84	7.85	7.28	0.57	L
0	3	34.37	36.15	1.78	5.17	4.91	0.25	L
0	3	35.67	38.63	2.97	8.32	7.68	0.64	L
0	3	35.91	39.98	4.08	11.35	10.19	1.16	L
0	3	34.98	41.12	6.13	17.54	14.92	2.62	D
0	3	34.98	41.12	6.13	17.54	14.92	2.62	D
0	3	38.22	45.00	6.78	17.74	15.06	2.67	D
4	2	33.53	35.35	1.82	5.42	5.14	0.28	L
4	2	33.29	34.94	1.65	4.96	4.73	0.23	L
4	2	34.11	39.76	5.65	16.57	14.22	2.36	D
2	2	37.13	40.29	3.17	8.53	7.86	0.67	L
2	2	38.30	38.41	0.11	0.28	0.28	0.00	L
2	2	39.29	39.18	-0.11	-0.29	-0.29	0.00	L
2	2	38.53	38.22	-0.31	-0.80	-0.80	0.01	L
0	2	38.06	45.00	6.94	18.23	15.42	2.81	D
0	2	35.46	40.87	5.41	15.26	13.24	2.02	D

Live cell concentration (log_{10})	Dead cell concentration (log_{10})	No treatment (Ct value)	Treatment (Ct value)	Treated – not treated (Ct value)	Change in Ct value not treated (%)	Change in Ct value treated (%)	Difference treated – not treated (%)	State of cells based on a 2.5 % cut-off value
0	2	38.06	45.00	6.94	18.23	15.42	2.81	D
0	2	43.85	45.00	1.15	2.61	2.55	0.07	D
0	2	41.97	45.00	3.03	7.22	6.74	0.49	D
0	2	41.57	45.00	3.43	8.25	7.62	0.63	D
0	2	39.71	45.00	5.29	13.31	11.75	1.56	D
0	2	42.10	45.00	2.90	6.90	6.45	0.45	D
0	2	40.79	45.00	4.21	10.32	9.36	0.97	D
1	1	35.70	38.33	2.63	7.37	6.86	0.51	L
1	1	35.91	38.98	3.07	8.55	7.88	0.67	L
1	1	35.70	39.20	3.50	9.80	8.93	0.88	L
3	1	35.71	36.21	0.51	1.42	1.40	0.02	L
3	1	34.48	34.66	0.19	0.54	0.53	0.00	L
3	1	33.75	37.54	3.79	11.22	10.08	1.13	L
3	1	33.46	34.33	0.87	2.59	2.53	0.07	L
6	0	27.26	28.18	0.92	3.38	3.27	0.11	L
6	0	26.58	27.81	1.24	4.65	4.44	0.21	L
6	0	25.78	28.75	2.98	11.54	10.35	1.19	L
5	0	29.79	31.03	1.24	4.15	3.99	0.17	L
5	0	26.95	27.85	0.97	3.60	3.47	0.13	L
5	0	29.92	30.68	0.76	2.55	2.49	0.06	L
5	0	26.95	27.92	0.97	3.60	3.47	0.13	L
5	0	29.13	32.19	3.06	10.50	9.51	1.00	L
4	0	32.54	33.40	0.86	2.66	2.59	0.07	L
4	0	30.16	30.76	0.63	2.11	2.06	0.04	L
4	0	30.16	30.79	0.63	2.11	2.06	0.04	L
4	0	33.40	33.00	-0.40	-1.19	-1.21	0.01	L
3	0	31.54	36.95	5.41	17.15	14.64	2.51	D
3	0	36.18	38.88	2.70	7.47	6.95	0.52	L
3	0	36.02	38.58	2.56	7.12	6.64	0.47	L
3	0	37.25	37.63	0.38	1.02	1.01	0.01	L
2	0	35.59	37.45	1.86	5.23	4.97	0.26	L
2	0	36.78	37.45	0.67	1.82	1.79	0.03	L
2	0	35.59	38.28	2.69	7.56	7.03	0.53	L
2	0	38.80	41.65	2.85	7.34	6.84	0.50	L
2	0	39.15	41.33	2.18	5.57	5.28	0.29	L
2	0	38.86	42.59	3.73	9.59	8.75	0.84	L
2	0	40.37	41.97	1.59	3.95	3.80	0.15	L
2	0	36.22	37.53	1.31	3.61	3.49	0.13	L

Live cell concentration (log_{10})	Dead cell concentration (log_{10})	No treatment (Ct value)	Treatment (Ct value)	Treated – not treated (Ct value)	Change in Ct value not treated (%)	Change in Ct value treated (%)	Difference treated – not treated (%)	State of cells based on a 2.5 % cut-off value
2	0	37.07	37.50	0.42	1.15	1.13	0.01	L
2	0	37.44	37.26	-0.18	-0.48	-0.48	0.00	L
2	0	36.26	37.43	1.17	3.23	3.13	0.10	L
2	0	40.56	45.00	4.44	10.95	9.87	1.08	L
2	0	40.94	42.45	1.50	3.67	3.54	0.13	L
1	0	45.00	41.70	-3.30	-7.34	-7.92	0.58	L
1	0	40.84	45.00	4.16	10.19	9.25	0.94	L
1	0	41.23	41.21	-0.02	-0.05	-0.05	0.00	L
1	0	41.82	41.43	-0.39	-0.94	-0.95	0.01	L
1	0	39.10	38.67	-0.43	-1.09	-1.10	0.01	L
1	0	41.15	40.33	-0.82	-2.00	-2.04	0.04	L
1	0	40.46	43.21	2.75	6.80	6.36	0.43	L
1	0	38.69	39.06	0.36	0.94	0.93	0.01	L

4.18 TESTING NATURALLY INFECTED SAMPLES

Yersinia ruckeri

Fish kidney tissues (*n* = 32) were subsampled and tested using the optimised protocol. Each homogenate was divided into four (100 µL each) and treated in the following way:

- - PEMAX dye.
- + PEMAX dye.
- Heat treated + PEMAX dye.
- Cultured for viability on BA.

Culture on BA showed that all samples, fresh and frozen, contained live *Y. ruckeri* cells. This confirms that samples that have been frozen and contain high numbers of *Y. ruckeri* may be used for vPCR. The limit of detection for these samples has not been assessed.

As all samples contained live *Y. ruckeri,* the % difference between PEMAX treated and untreated samples was expected to be ≤ 1 % (i.e., indicating the presence of live cells in the sample). The % difference values between PEMAX treated and untreated heat killed samples were expected to be > 1 %, as there should only be dead cells in the sample.

Results showed that for + PEMAX dye samples, there was an average Ct difference between qPCR – v-qPCR of 0.53 with a SD of 0.87. Sample number four did not conform to the proposed cut-off value of ≤ 1 % for samples that contain live cells. The Ct value of the v-qPCR assay for this sample was 35.13, which is past the cut-off value of 32. An enrichment step for samples that contain low concentrations of *Y. ruckeri* may assist in determining the viability of cells within the sample. However, as testing aquatic animals for the presence of pathogens is generally carried out on multiple animals representing the

population, one undetermined result would not affect the outcome that there are live cells present in the population (OIE, 2016).

There was an average Ct difference of 7.64 (± 2.5 SD) between heat treated v-qPCR samples and the qPCR results (Table 28). Three heat killed samples did not conform to the proposed cut-off value of > 1 %, (i.e., samples 2, 3 and 4). Two of these samples were above the cut-off value of 32 of the assay and one was close (31.06). An enrichment step may be required in these cases. Note that for operational samples, the heat treatment sample will not be included.

All conventional PCR results were positive for + PEMAX dye, - PEMAX dye and heat treated + PEMAX dye samples apart from sample 10, heat treated + PEMAX dye. All of the heat treated samples showed a weaker amplicon than the other two samples. This concludes that this conventional PCR is not appropriate for use with vPCR for *Y. ruckeri* and will not give full suppression.

Table 28: Summary of results from naturally infected fish tissue samples processed with and without PEMAX treatment in qPCR and culture on agar. Red = samples that gave unexpected results.

Sample	qPCR (Ct value)	v-qPCR (Ct value)	Heat treated v-qPCR (Ct value)	qPCR – v-qPCR	qPCR – heat treated v-qPCR	% difference (heat treated)	% difference (dye treated)	Culture positive
1	18.69	18.19	27.55	-0.5	8.86	15.25	0.07	✓
2	31.06	29.67	34.1	-1.39	3.04	0.03	0.21	✓
3	33.45	33.91	33.73	0.46	0.85	0.01	0.02	✓
4	32.25	35.13	35.26	-2.88	-3.01	0.73	0.80	✓
5	23.27	23.56	34.23	0.29	10.96	15.08	0.02	✓
6	19.89	20.07	30.07	0.18	10.18	17.33	0.01	✓
7	23.41	23.55	34.06	14	10.65	14.23	0.00	✓
8	23.67	24.36	34.56	0.69	10.89	14.50	0.08	✓
9	28.18	29.31	34.09	1.13	5.91	3.64	0.15	✓
10	24.85	25.2	35.13	0.35	10.28	12.11	0.02	✓
11	17.96	17.92	28.35	-0.04	10.39	21.20	0.00	✓
12	25.49	25.43	34.23	-0.06	8.76	8.79	0.00	✓
13	26.25	25.9	31.3	-0.36	5.04	3.10	0.02	✓
14	25.17	25.63	31.15	0.47	5.99	4.57	0.03	✓
15	26.11	26.35	32.79	0.24	6.69	5.22	0.01	✓
16	24.73	24.85	30.19	0.11	5.46	4.00	0.00	✓
17	24.84	25.32	31.18	0.48	6.34	5.19	0.04	✓
18	19.91	21.82	29.74	1.9	9.82	16.30	0.83	✓
19	21.94	24.06	31.19	-0.28	6.85	12.51	0.85	✓
20	18.89	20.16	29.36	1.27	10.47	19.76	0.42	✓
21	22.44	23.71	30.38	1.27	7.93	9.23	0.30	✓
22	19.18	20.08	28.99	0.9	9.8	17.28	0.21	✓
23	21.56	22.72	31.1	1.17	9.54	13.58	0.28	✓
24	21.04	21.32	29.01	0.28	7.97	10.41	0.02	✓
25	22.68	23.46	27.66	0.78	4.98	3.95	0.11	✓
26	22.17	23.61	32.05	1.43	9.88	13.75	0.40	✓
27	22.72	23.38	29.53	0.66	6.81	6.92	0.08	✓
28	23.34	23.73	29.13	-0.38	5.79	4.93	0.03	✓
29	22.97	24.45	29.5	1.47	6.53	6.29	0.39	✓
30	19.06	19.86	28.4	0.8	9.34	16.11	0.17	✓
31	21.49	22.91	29.68	-1.41	8.18	10.48	0.40	✓
32	23.89	25.29	28.88	1.4	4.99	3.60	0.32	
Average	-	-	-	0.53	7.64	-	-	-
SD	-	-	-	0.87	2.52	-	-	-

Tenacibaculum maritimum

Skin lesions (*n* = 30) were sub-sampled and tested with the optimised protocol using conventional PCR, qPCR and cultured on AO + sea salt agar. Culture was carried out using a neat sample of the homogenate (also used for PCR, vPCR and v-qPCR) as well as on diluted homogenates (10^{-1}, 10^{-2}) to ensure the best chance of detection in a background of environmental isolates. Aliquots (100 µL) of neat and the dilutions were spread plated onto the agar and incubated at 22 °C for 7 d.

Two samples were confirmed to contain live *T. maritimum* by culture. However, as the plates from all samples contained a large amount of growth from environmental bacteria, it is possible that low levels of *T. maritimum* may not have been detected.

The % Δ Ct of the heat treated + PEMAX dye samples should be > 2.5 %, indicating that only dead cells are present in the sample. The % Δ Ct for the + PEMAX samples should be ≤ 2.5 % for the two samples positive by culture.

Of the untreated samples, 23 produced an amplification curve with an average Ct value of 37.09 in the qPCR and 16 samples produced an amplicon in the conventional PCR.

For the PEMAX treated samples, 11 produced an amplification curve with an average Ct value of 37.97 in the v-qPCR and 3 produced an amplicon in the conventional vPCR. The results could not reliably be interpreted for the majority (8/11) of the v-qPCR positive samples as they were below the detection limit of the assay (Ct value > 36).

For the heat treated and PEMAX treated samples, 11 produced an amplification curve with an average Ct value of 37.94 in the qPCR and no samples produced an amplicon in the conventional PCR.

Using the indicative % Δ Ct value, seven of 11 PEMAX treated samples had a value of ≤ 2.5 % indicating live cells were present. Only three of the PEMAX treated samples were within the LOD of the assay (Ct value < 36). Of these samples (samples 16, 25 and 27), all % Δ Ct values were ≤ 2.5 % indicating live cells were present. Two of these three samples (i.e., samples 16 and 27) were verified as live by culture.

The heat killed PEMAX treated samples all had a % Δ Ct value of > 2.5 % (Table 29).

Samples at or below the LOD are not ideal for assay validation, however, they may be representative of naturally infected samples. For these low concentrations, previous experiments have shown that the assay at or below the LOD is not robust, which is likely to account for the inconsistent results. An enrichment protocol may help to verify v-qPCR results.

Table 29: Summary of results from naturally infected skin ulcer tissue samples processed with and without PEMAX treatment by conventional PCR, qPCR and culture on agar.

Sample	qPCR (Ct value)	v-qPCR (Ct value)	Heat treated v-qPCR (Ct value)	qPCR – v-qPCR	qPCR – heat treated v-qPCR	% difference (heat treated)	% difference (dye treated)	Conventional PCR	Conventional v-PCR	Heat killed vPCR	Culture positive
1	37.75	39.67	39.28	-1.92	0.39	3.95	0.25	✓	✗	✗	✗
2	40.95	-	-					✓	✗	✗	✗
3	42.25	-	-					✗	✗	✗	✗
4	35.32	37.81	39.51	-2.49	-1.7	11.56	0.46	✓	✗	✗	✗
5	-	-	-					✗	✗	✗	✗
6	-	-	-					✗	✗	✗	✗
7	30.43	36.58	37.16	-6.15	-0.58	21.52	3.40	✓	✗	✗	✗
8	39.12	-	-					✓	✗	✗	✗
9	32.02	38.28	37.2	-6.26	1.08	15.74	3.20	✓	✗	✗	✗
10	-	-	-					✗	✗	✗	✗
11	33.03	36.2	37.45	-3.17	-1.25	13.02	0.84	✓	✓	✗	✗
12	-	-	-					✗	✗	✗	✗
13	40.81	-	-					✗	✗	✗	✗
14	35.81	40.54	42.14	-4.73	-1.6	17.26	1.54	✓	✗	✗	✗
15	39.86	-	-					✓	✗	✗	✗
16	30.37	33.02	35.25	-2.65	-2.23	15.61	0.70	✓	✓	✗	✓
17	30.81	39.16	37.34	-8.35	1.82	20.63	5.78	✓	✗	✗	✗
18	42.61	-	-					✗	✗	✗	✗
19	42.62	-	-					✗	✗	✗	✗
20	-	-	-					✗	✗	✗	✗
21	39.68	-	-					✗	✗	✗	✗

Sample	qPCR (Ct value)	v-qPCR (Ct value)	Heat treated v-qPCR (Ct value)	qPCR – v-qPCR	qPCR – heat treated v-qPCR	% difference (heat treated)	% difference (dye treated)	Conventional PCR	Conventional v-PCR	Heat killed vPCR	Culture positive
22	41	-	-					✗	✗	✗	✗
23	-	-	-					✗	✗	✗	✗
24	-	-	-					✗	✗	✗	✗
25	29.64	33.05	33.31	-3.41	-0.26	12.01	1.19	✓	✗	✗	✗
26	43.68	-	-					✗	✗	✗	✗
27	32.07	35.85	37.87	-3.78	-2.02	17.61	1.24	✓	✓	✗	✓
28	33.13	38.82	40.83	-5.69	-2.01	22.67	2.52	✓	✗	✗	✗
29	38.65	-	-					✓	✗	✗	✗
30	41.5	-	-					✓	✗	✗	✗
Average	37.09	37.18	37.94	-4.42	-0.76						
SD	4.61	2.40	2.35	1.90	1.31						

A second batch of skin tissue samples were received in February 2019 for validation of the protocol with the addition of the enrichment protocol described below (section 4.19). Thirty five samples were received however only 13 of these samples contained *T. maritimum* DNA as tested by the qPCR. The results from these 13 samples are described (Table 30).

Skin lesions were sub-sampled and tested with the optimised protocol using conventional PCR, qPCR and cultured on AO + sea salt agar. Culture was carried out using a neat sample of the homogenate (also used for PCR, vPCR and v-qPCR) as well as on diluted homogenates (10^{-1}, 10^{-2}) to ensure the best chance of detection in a background of environmental isolates. Aliquots (100 µL) of neat and the dilutions were spread plated onto the agar and incubated at 22 °C for 7 days.

For the PEMAX treated samples, 10 of 13 produced an amplification curve with an average Ct value of 34.82 in the v-qPCR and eight produced an amplicon in the conventional vPCR. The results could be reliably be interpreted for nine of the 13 samples as they were within range of the assay (i.e., Ct values < 36). Using the indicative % Δ Ct value for these samples, all nine PEMAX treated samples had a value of ≤ 2.5 % indicating live cells were present. Three of these nine samples were confirmed to contain live *T. maritimum* cells by culture. As with the initial naturally infected samples, it is possible that *T. maritimum* colonies may have been missed due to the overgrowth from environmental bacteria. The Ct values from the samples where *T. maritimum* was isolated were 25, 36 and 28.

Heat and PEMAX treated samples revealed eight of nine samples could be reliably analysed and produced an amplification curve with an average Ct value of 38.20 in the qPCR. Two of these nine samples produced a very weak amplicon in the conventional PCR. These were from two samples with the lowest Ct values; 25 and 27. These values are out of range for the conventional nested PCR as the concentration of *T. maritimum* in the sample was < 10^3 CFU mL^{-1}. Using the indicative % Δ Ct value for all samples that could be reliably analysed, two samples produced unexpected results of < 2.5 %.

Enrichment protocol:

Enrichment was carried out on the seven samples that were unable to be reliably assessed in the first step. Due to logistical constraints, these samples could only be enriched for 24 hours. This reduced enrichment time may have compromised the test results. After enrichment, all samples were still below the limit of the assay (i.e., Ct value > 36). Three samples had a Ct value after v-qPCR which may be indicative of growth. One enriched sample had a Ct value that remained the same which may indicate that the cells are live but not actively replicating. All other samples had a higher Ct value than the original v-qPCR equating to at least 1 log reduction in growth, likely indicating these cells were dead although one of these samples did produce a positive culture result on initial processing (Ct value > 40). Reliable conclusions cannot be drawn from this experiment as the sample number is very low.

Table 30: Summary of results from naturally infected skin ulcer tissue samples that contained *T. maritimum* DNA and were processed with and without PEMAX treatment by conventional PCR, qPCR and culture on agar. Red = samples that gave unexpected results. E = enrichment, * = enriched for 24 hours only.

Sample	qPCR (Ct value)	v-qPCR (Ct value)	Heat treated v-qPCR (Ct value)	qPCR – v-qPCR	qPCR – heat treated v-qPCR	% difference (heat treated)	% difference (dye treated)	Conventional PCR	Conventional v-PCR	Heat killed vPCR	Culture positive
31	39.27	39.56	-	-0.29	NA	0.01	NA	✓	✗	✗	✗
32	38.45	-	-					✓	✗	✗	✗
33	30.69	34.81	35.67	4.12	4.68	2.02	1.59	✓	✓	✗	✗
34	25.4	25.54	30.17	0.14	2.76	2.97	0.00	✓	✓	very weak	✓
35	32.48	35.78	38.38	3.3	5.9	2.79	0.94	✓	✓	✗	✗
36	39.43	-	-					✓	✗	✗	✗
37	38.71	-	-					✓	✗	✗	✗
38	35.48	38.19	42.43	2.86	7.38	2.29	0.54	✓	✓	✗	✗
39	36.81	39.67	43.86	2.86	7.05	2.78	0.56	✓	✓	✗	✓
40	35.17	41.26	43.76	6.09	8.59	4.22	2.15	✓	✗	✗	✗
41	35.83	35.68	-	0.15			0	✓	✓	✗	✗
42	28.61	29.93	32.52	1.32	2.46	1	0.2	✓	✓	✗	✓
43	27.64	27.75	38.79	0.36	11.4	6.62	0.02	✓	✓	very weak	✗
31E	40.68	44.13	-	3.45			0.66	✓	✗	✗	✗
32E	39.82	43.37	-	3.55			0.73	✓	✗	✗	✗
36E*	41.18	-	-	NA			NA	✗	✗		✗
37E*	38.32	40.04	-	1.72			0.19	✓	✗		✓
38E*	38.37	38.48		0.11			0.00	✓	✗		✗
39E*	39.29	44.05		4.76			1.31	✓	✗		✗
40E*	44.91	-		NA			NA	✓	✗		✗

4.19 ENRICHMENT PROTOCOL FOR *TENACIBACULUM MARITIMUM*

For *T. maritimum*, false negative v-qPCR results were likely (i.e., ≤ 2.5 % difference; Figure 44) when samples contained a low concentration (< 10^4 CFU mL-1) of live *T. maritimum* cells in the background of high concentration of dead cells, and the samples were processed without enrichment. The enrichment protocol carried out on the same samples, improved the results and provided a repeatable cut-off value. This was shown by processing independent replicates (*n* = 3) on a variety of samples with a mix of live and dead cells.

In addition, the increase in the Ct value in the enriched samples compared to the samples processed immediately when live cells were present could be used as an indicator for viability. Conversely, a decrease the Ct value could be seen in the samples processed immediately compared to enriched samples when the samples contained only dead cells. This was likely due to the degradation of the DNA and not a dilution of the pathogen as the same volume is used for each process.

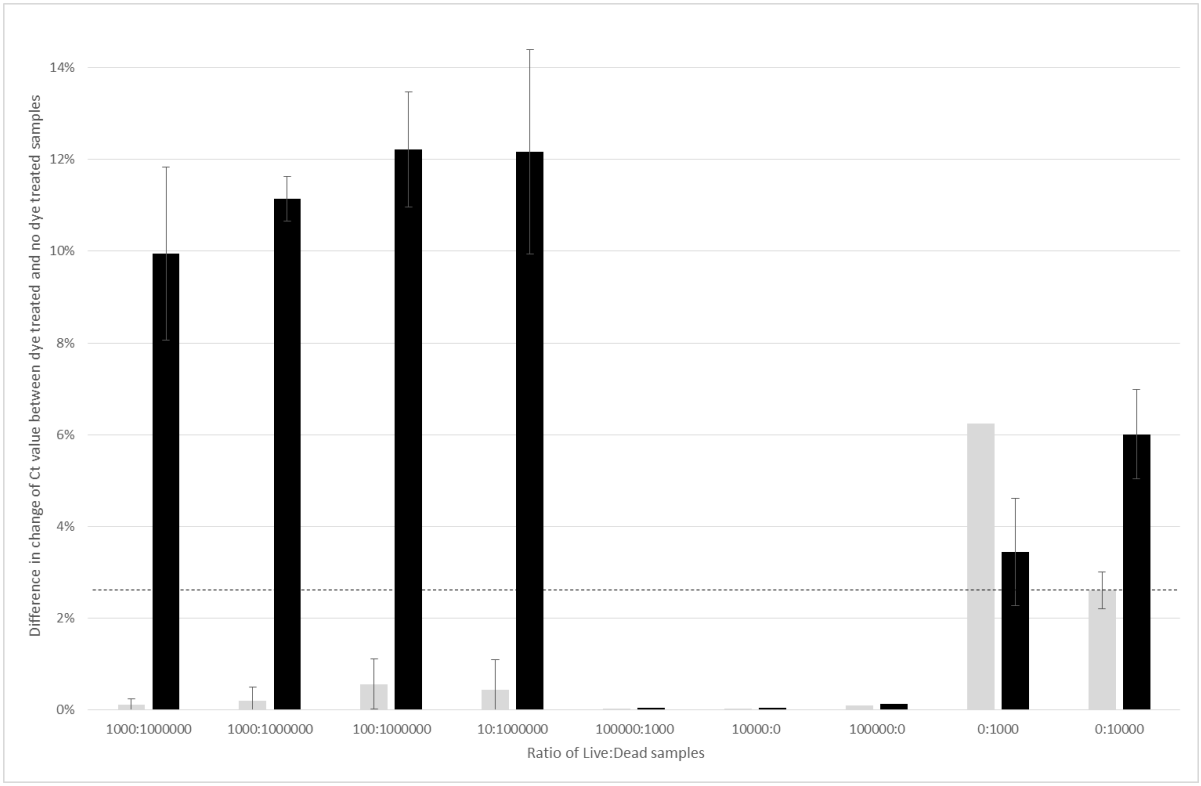

Figure 44. Comparison of % difference between samples processed immediately and processed after enrichment. Dashed line = cut-off value for cells that are live (2.5 %). *n* = 3. Samples with no error bars, only had one replicate amplify in the v-qPCR.

The first batch of samples from the field were processed immediately (Section 4.17) and then frozen at -20 °C for storage. The stored samples were then defrosted and tested with the enrichment protocol to determine if *T. maritimum* could remain viable after freezing and be detectable by v-qPCR.

Once defrosted, 300 µL of the homogenate was inoculated into 3 mL TYG-M broth and incubated at 22 °C for 48 h. The samples were then processed as above (section 4.18) and the results were compared

to the previous results (Section 4.17). An increase in all Ct values (data not shown) indicated that the frozen samples did not contain live *T. maritimum*. This is useful information to understand that tissues infected with *T. maritimum* should not be frozen at -20 °C prior to vPCR testing.

This protocol will need to be trialled on an increased number of fresh field samples to verify the results obtained from artificially spiked tissue.

5 Summary

This study showed the relative importance of the tested parameters during the optimisation and validation of vPCR for different aquatic pathogens (Table 31).

Table 31: Summary of the optimisation parameters used during the present study and their importance for use in future optimisation.

Parameter	Include in optimisation	Comments
Limit of detection	✓	To be determined once the optimal parameters have been assessed. To be carried out on live cells with and without a background of dead cells.
Dye concentration	✓	Only to be carried out on a high concentration of the target cells for optimisation.
Dye incubation times	×	Use a default time of 15 min.
Incubation temperature	✓	
Photoperiod	×	Use a default time of 15 min.
Dye type	×	Use PEMAX for optimisation. Only use EMA or PMA if PEMAX doesn't provide desired results.
Washing steps	×	
Resuspension buffers	✓	Showed differences for some target cells and should be included in initial optimisation.
Double dye	✓	
Double tube	×	
Plastic-ware	✓	Use specific vPCR tubes or clear 96 well Lo-bind plastic plates.
Artificially infected tissue	✓	Use to ensure matrix will not interfere with the protocol.
Enrichment	✓	Use for target cells past the limit of detection of the assay.
Naturally infected tissues	✓	It is important to test the optimised protocol on naturally infected tissue to ensure it performs as for artificially infected tissue.

6 Discussion

Rapid methods to detect live cells have many purposes critical to informing biosecurity decision making including: decisions about a pathogen incursion, differentiation between vaccine strains (dead cells) from an infection, and to provide information on the level of live cells present to determine the risk of infection. The use of traditional culture methods for some aquatic pathogens to assist decision making can be problematic due to their fastidious nature, overgrowth by faster growing environmental organisms in the sample or organisms being in a viable but non-culturable state, for example, *Tenacibaculum maritimum*. This is particularly so if the level of infection is low.

This proof-of-concept study represents the first time vPCR has been optimised and validated to detect live aquatic animal pathogens. For the pathogens examined (*Yersinia ruckeri*, *T. maritimum*, Aquabirnavirus), the optimised protocols were shown to be robust and transferable with different users producing equivalent results. Importantly, this study assessed the suitability of v-qPCR and vPCR to identify live pathogens in different sample matrices, including in skin tissue where environmental bacteria are expected to be present in relatively high numbers. The optimisation and validation undertaken in this study highlights the importance of robust processes to ensure that the technology is fit-for-purpose with appropriate sensitivity and reliability for adoption in a regulatory setting.

The following factors were considered for assay optimisation: dye concentration (including double dye exposure); dye incubation periods; photoperiod; dye incubation temperature; the use of reaction buffers; washing steps (*Y. ruckeri* only); and the use of a double tube method (*Y. ruckeri*).

For *Y. ruckeri*, dye concentration, double dye exposure, incubation temperature and the use of a different resuspension buffers were the only parameters that made a significant improvement for v-qPCR. For *T. maritimum* dye concentration was the only parameter to have any significant influence on v-qPCR and ABV, dye concentration and incubation temperature were the two parameters that had a significant influence on RT-PCR.

Dye concentration was found to be an important parameter to optimise the vPCR assays for each pathogen studied. Both PEMAX and EMA were more efficient when used at a high concentration (100 µM), however for *T. maritimum* increasing the dye concentration above 50 µM did not improve the assay. The different dye concentrations necessary for each of the pathogens was likely due to differences in ability of the dye to penetrate their cell walls or membranes. For *Y. ruckeri* and *T. maritimum*, PEMAX and EMA dye concentrations showed very similar results. For *Y. ruckeri* exposed to a double dose of PEMAX resulted in a significantly improved results. By contrast, assay improvement was not seen when *T. maritimum* was exposed to a double dose of PEMAX. EMA was not more efficient when a double dye amount was used in either *Y. ruckeri* or *T. maritimum*. This difference may be due to the concentrations of EMA assessed in the current study. Low amounts of EMA as a double dose have shown to be effective for *Enterobacter sakazakii* (Minami et al., 2010) so a wider variety of concentrations tested may have yielded different results. For ABV, EMA was less efficient than PEMAX and complete suppression was not seen even when used at 100 µM. Importantly, it was found that none of the concentrations of PEMAX

dye that were optimised were able to penetrate the cell wall and membrane of live bacteria and virus and subsequently impair the amplification of nucleic acid.

Efficiency of vPCR was not improved under different incubation times or different photoperiods for any pathogen evaluated. However, sample incubation times may require further consideration for application on other pathogens due to differences in cell walls or membranes i.e., Gram-positive bacteria or protozoan parasites. The photoperiods administered in the present study did not improve vPCR efficiency. The original light source used for vPCR was halogen bulbs (Rawsthorne et al 2009). This light source was not standardised and the effective time of exposure was dependent on the distance of the tube to the light source. It is noted that the heat provided by the halogen source could also affect cell walls or membranes and thus the effectiveness of vPCR. The two light platforms used in this study, PhAST and PAUL, were LED based and were standardised with all samples being equal distance from light sources that do not produce heat. The results from this study suggest that using LED light sources negates optimisation beyond the default time of 15 min.

Incubation temperature was shown to have an impact on efficiency for *Y. ruckeri* and ABV but not for *T. maritimum*. Differences in efficiency between the incubation temperatures used on different pathogens is likely due to changes in the pathogens membrane fluidity at the different temperatures which may affect dye penetration. The results for *Y. ruckeri, T. maritimum* and ABV differ from other pathogens studied where higher incubation temperatures significantly improved assay efficiency (Nkuipou-Kenfack et al., 2013) highlighting the importance of optimising this parameter for each new pathogen examined. While temperature did not improve the efficiency of the RT-PCR assay for ABV, higher incubation temperatures did reduce the intensity of the amplicon produced in the RT-PCR. This reduction in intensity is likely due to the degradation of RNA prior to lysis, as RNA is less stable at higher temperatures. For *T. maritimum* a negative impact on live treated cells was observed at 4 °C compared with live treated cells on ice. The different results at the different temperatures was unlikely to be genuine as this trend was not consistent among the other temperatures examined.

The addition of reaction buffers produced variable results. Triton X at a concentration of 0.1 % improved the *Y. ruckeri* vPCR while none of the buffers evaluated improved the *T. maritimum* vPCR. The results from the *T. maritimum* vPCR are in contrast to other studies where the addition of reaction buffers significantly improved vPCR efficiency (Codony et al., 2015, Takahashi et al., 2017). In the present study, some reaction buffers either did not improve the assay or resulted in lowered effectiveness, for example, re-suspension in seawater or broth. The effectiveness of ethidium bromide, the chemical EMA is derived from, is known to be influenced by the presence of salt by either competition between the sodium ions and the dye molecules (Graves et al., 1981) or due to osmotic shock of the salinity (Shi et al., 2011). Interestingly, no reductions in efficiency were observed when processing artificially infected skin tissue samples of fish originating from seawater compared to pure culture. However, the protocol may have to be altered to include washing to remove the salts if the assay was applied to detect *T. maritimum* in seawater. Buffers should be evaluated for any new pathogen or matrix to improve the suppression of amplicons from dead cells.

The use of washing steps to remove debris from the sample prior to dye treatment reduced the efficiency of *Y. ruckeri* vPCR. This reduction may be due the washing and centrifugation process changing the structure of the cell wall and membrane thus reducing the dye penetration (Peterson et al., 2011). Using double tubes also provided no improvement on the vPCR. Amber tubes were shown to reduce the efficiency of vPCR as bacterial cells remained in the tube and were not accessible by the dye. This finding is consistent with other reports (Agusti et al., 2017).

The limit of detection (LOD) of the viability assay was determined for each pathogen examined. Test sensitivity is extremely important for interpretation of the results. The *Y. ruckeri* v-qPCR is not reliable at bacterial concentrations of < 10^4 CFU mL^{-1} or a cut off of Ct value of 32. However, as *Y. ruckeri* causes a bacterial septicaemia and infected fish are likely to have a heavy pathogen burden, this high LOD may not be a cause for concern. Additionally, aquatic animals should be tested at high enough numbers to deduce a population prevalence of a pathogen (OIE, 2016), therefore the detection of one fish with live pathogens may be enough to determine or inform a decision depending on the reason for testing. Of the 32 samples tested that were naturally infected with *Y. ruckeri,* only one was below the LOD of the assay providing further confidence that a LOD of Ct 32 is appropriate for this pathogen. This high LOD may be due to the size of the PCR product as it has been shown that vPCR is more efficient when longer PCR products are used. Optimisation of a *Y. ruckeri* PCR with a longer product size may resolve this loss of sensitivity. The LOD for the ABV RT-PCR was also relatively high at 10^4 $TCID_{50}$. The LOD was shown to be inadequate for detection of low levels of virus ABV in spiked tissue but is sufficient for detection of ABV from cell culture material.

Conventional vPCR for *T. maritimum* and conventional RT-PCR for ABV showed complete suppression of amplicons from dead treated cells. It is documented that longer PCR products result in better or complete suppression of amplicons (Banihashemi et al., 2012, Seidel et al., 2017). This is due to the dye binding in a certain stoichiometry. That is, the probability that a binding event has occurred and inhibition of amplification during PCR is higher when longer amplicons are amplified. The *T. maritimum* vPCR assay showed complete suppression in samples that contained only dead cells when the bacterial concentrations were ≤ 10^6 CFU mL^{-1} and complete suppression was observed in samples containing ABV cells at all concentrations evaluated. Complete suppression was not seen with the *Y. ruckeri* vPCR and this PCR assay was not carried out on all samples. The *Y. ruckeri* conventional PCR amplicon size was half the size of *T. maritimum* and ABV vPCR which may have contributed to incomplete suppression in this assay.

The *T. maritimum* v-qPCR had a reduced amplification efficiency with live cells diluted in a background of dead cells. This could be due to the carryover of dye or dead cell debris from the sample interfering with the PCR reaction. However, this is unlikely to affect the results of v-qPCR for detection of live *T. maritimum* if the concentration of bacteria is above the LOD. As the concentration of pathogens is unknown prior to testing the sample, it is recommended to use the enrichment protocol as a standard method. The linear range and LOD for v-qPCR and vPCR of *T. maritimum* in the present study is consistent with those of previous studies (Dinu et al., 2012, Maće et al., 2013, Thanh et al., 2017,

Daranas et al., 2018). Similar to Fittipaldi et al., (2012) this study found that v-qPCR for live cell concentrations of < 10^3 CFU mL^{-1} in the presence of a high number of dead cells was unreliable. Although, vPCR for *T. maritimum* has shown to be repeatable and reproducible, it was shown to be less reliable than v-qPCR in artificially spiked samples at low bacterial concentrations. Additionally, conventional PCR is time consuming and does not lend itself to high-throughput processes. Furthermore, nested conventional PCR has an inherent risk of contamination which may produce inaccurate results. For these reasons, a v-qPCR protocol is preferred and was optimised in the present study.

Testing the v-qPCR on naturally infected tissue was possible for *Y. ruckeri* and *T. maritimum* only. Testing on *Y. ruckeri* infected tissue demonstrated a high degree of repeatability. Samples tested generally contained *Y. ruckeri* at a high abundance with all but one sample being within the cut-off range of the v-qPCR. All samples were shown to be live by culture and by using the indicative % Δ Ct value. Three samples that were heat treated then treated with dye did not conform to the indicative % Δ Ct value. These samples had the highest Ct values and were at the LOD of the assay. The reason for this discrepancy is unknown however variable results were observed with decreased bacterial concentrations. An enrichment step or performing the assay on triplicate samples may help to resolve such inconclusive results.

Performing v-qPCR and vPCR on *T. maritimum* naturally infected tissues demonstrated a lower likelihood of overestimating the infectivity of *T. maritimum* in the sample compared to using qPCR and PCR. The high presence of dead cells within these samples could be an artefact of field sampling leading to cross contamination, or it could be an accurate result of dead cells within the skin lesion. Selection of the piece of tissue for testing can affect analysis of pathogen viability as the target pathogen may no longer be live in the centre of the lesion and secondary bacteria are more likely to be present (Buller 2014). Therefore skin lesions should be sampled from the leading edge as the target pathogen is most likely to be live and invading the tissue. Thirty tissue samples were tested for the presence of live *T. maritimum*. Of those samples, 23 had *T. maritimum* DNA and of those 23, three were within the LOD of the assay (i.e., v-qPCR < Ct 36) and could be reliably analysed. These three samples were found to contain live cells based on the % Δ Ct and two of them were confirmed positive by culture. Detection by culture from these samples proved difficult with heavy mixed environmental growth therefore, it is possible that the culture result for the negative sample was inaccurate. A second batch of tissues were processed with the addition of an enrichment step. Thirty five samples were received with 13 of these containing *T. maritimum* DNA and six being within the LOD of the assay. These six samples had a % Δ Ct of < 2.5 and two were found to contain live cells by culture. Another sample that had live cells by culture (and had a % Δ Ct of < 2.5) had a Ct value below the LOD of the assay so could not be reliably analysed.

Analysis of seven of 13 *T. maritimum* infected samples that could not be reliably analysed prior to enrichment, showed that the cells were not replicating as fast as they were for the artificially spiked samples. For some samples, the Ct values remained the same or varied by approximately 1 Ct. This may indicate that the cells have entered into a culturable but non-viable state. It could be that the cells

in this sample are suspended in the fish mucus until more favourable conditions become apparent and that the length of incubation in the media was not enough to activate them even in a laboratory environment. Extending the incubation time may help with understanding this relationship, if the DNA concentration remains the same after a longer incubation it is more likely these cells are in a viable but non-culturable state rather than being dead. Alternatively, it may be that the rate of degradation of *T. maritimum* cells from naturally infected fish differs from cells maintained in the laboratory. Again a longer incubation period would help to clarify this.

The survivability of *T. maritimum* may be an important in assisting our understanding of these results. In sterile media, survivability can be for extended periods of time, however in non-sterile seawater this time can be reduced to approximately five days and the pathogen does not remain culturable (Avendaño-Herrera et al., 2006a). This is likely to hinder the ability to reliably culture this organism in the laboratory and may account for differences seen in the naturally infected compared to artificially infected samples. Much is still unknown about this organism including its transmission and survival strategy. For example, if this organism cannot survive for extended periods in non-sterile seawater, what is the mechanism for horizontal transmission? Using v-qPCR may help to differentiate between viable but non-culturable cells compared to dead cells to assist understanding of survival strategies and therefore help researchers elucidate the route of infection.

For ABV, preliminary results from artificially spiked tissues showed that the tissue matrix did not interfere with assay performance. This shows the potential to use RT-PCR on viral particles, however testing on naturally infected samples is required before the assay could be adopted for routine diagnostic purposes in a regulatory setting. The ABV RT-PCR assay was shown to have limited sensitivity when testing infected tissues and should only be used for material from cell culture.

The use of high-throughput methods for *T. maritimum* and ABV were shown to be efficient and transferrable, allowing for 96 samples to be processed at one time. As diagnostic samples from aquatic animals are tested in a sample size to have confidence in the results; i.e. more than one animal, having high-throughput capability allows for more timely reporting of results.

7 Conclusion

The optimisation and validation of vPCR to discriminate between viable and non-viable target organisms provides MPI with the ability to make timely decisions.

This proof-of concept study highlights the potential for vPCR to be used to detect live *T. maritimum* and *Y. ruckei* within salmon tissue. For *T. maritimum* vPCR has shown to be reliable to detect live cells from skin tissue at concentrations above 10^3 CFU mL^{-1}. An enrichment protocol is recommended to allow detection of low numbers of live *T. maritimum* cells with and without a background of high concentration of dead cells. For *Y. ruckeri* this detection has been shown to be reliable to detect live cells from kidney tissue at concentrations above 10^4 CFU mL^{-1}.

This study has also shown the potential for vPCR to be used on viral particles however this assay requires further validation on naturally infected samples prior to use in a diagnostic laboratory.

It is recommended to use vPCR on multiple animals to determine the presence of live pathogens in the population rather than on individual fish. Alternatively, multiple pools of tissue from the same animal should be tested. This is likely to help clarify any inconclusive results from low levels of pathogen in the sample.

Where optimised the vPCR can be transferred to a 96 well plate format, high-throughput processing to increase efficiency.

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9 Appendices

9.1 APPENDIX 1 – *YERSINIA RUCKERI* PROFICIENCY TESTING

Method:

Add 1 mL 0.1 % Triton X-100 to each of the 20 x 100 mg sample in the bioreba bag or 2mL safelock tube.

Homogenate sample by macerating until homogenous if using the bioreba bags or place in the Tissue lyser II and homogenise at 15 Hz for 30 sec.

Remove 100 µL of the homogeneous solution into 2 separate vPCR tubes (X.1, X.2 in duplicate).

Add 5 µL PEMAX dye* to X.1 in a BSC in the dark.

Vortex and incubate all tubes (X.1 and X.2) in the dark on ice immediately on shaking platform at 200 rpm.

Incubate for 15-30 min.

Centrifuge tubes for 3 min at 10, 000 x g.

Remove and discard supernatant and re-suspend pellet in 100 µL saline.

Mix until a homogeneous solution is formed and transfer to a 96-well plate in layout as below.

1.1	2.1	3.1	4.1	5.1	6.1	7.1	8.1	9.1	10.1	11.1	12.1
1.2	2.2	3.2	4.2	5.2	6.2	7.2	8.2	9.2	10.2	11.2	12.2
13.1	14.1	15.1	16.1	17.1	18.1	19.1	20.1				
13.2	14.2	15.2	16.2	17.2	18.2	19.2	20.2				

Add 5 µL PEMAX dye to all X.1 in a BSC in the dark and mix carefully 3 x by repeat pipetting with a 100 µL pipette.

Seal the plate and incubate in the dark on ice immediately on a shaking platform at 200 rpm.

Incubate for 15-30 min.

Place the 96-well plate on PAUL and expose to light at 100 % intensity for 15 min (default setting).
After incubation add 180 µL ATL and 20 µL proteinase K to each sample, mix carefully and transfer to a screw cap tube.

Incubate at 56 °C overnight.

Following lysis, add 100 µL VXL mixing immediately. Incubate at 65 °C for 10 min, remove from PC3 and extract on the QIAcube or manually using the QIAamp mini DNA kit or similar using standard protocols and eluting in 200 µL.

Run all samples in the real time PCR in duplicate.

***PEMAX is a potential carcinogen, use nitrile gloves when handling it and dispose of waste as cytotoxic.**

Sample	Sample number	Dye	Cell concentration added	Final cell concentration
1	**1.1**	Yes	10e9 L	10e8 L
	1.2	No	10e9 L	10e8 L
2	**2.1**	Yes	10e9 D	10e8 D
	2.2	No	10e9 D	10e8 D
3	**3.1**	Yes	10e8 L 10e6 D	10e7 L 10e5 D
	3.2	No	10e8 L 10e6 D	10e7 L 10e5 D
4	**4.1**	Yes	10e7 D	10e6 D
	4.2	No	10e7 D	10e6 D
5	**5.1**	Yes	10e7 L	10e6 L
	5.2	No	10e7 L	10e6 L
6	**6.1**	Yes	10e6 L	10e5 L
	6.2	No	10e6 L	10e5 L
7	**7.1**	Yes	10e6 D	10e5 D
	7.2	No	10e6 D	10e5 D
8	**8.1**	Yes	10e6 L 10e6 D	10e5 L 10e5 D
	8.2	No	10e6 L 10e6 D	10e5 L 10e5 D
9	**9.1**	Yes	10e5 D	10e4 D
	9.2	No	10e5 D	10e4 D
10	**10.1**	Yes	10e5 L	10e4 L
	10.2	No	10e5 L	10e4 L
11	**11.1**	Yes	10e9 L 10e9 D	10e8 L 10e8 D
	11.2	No	10e9 L 10e9 D	10e8 L 10e8 D
12	**12.1**	Yes	10e7 D	10e6 D
	12.2	No	10e7 D	10e6 D
13	**13.1**	Yes	10e7 L 10e5 D	10e6 L 10e4 D
	13.2	No	10e7 L 10e5 D	10e6 L 10e4 D
14	**14.1**	Yes	10e8 D	10e7 D
	14.2	No	10e8 D	10e7 D
15	**15.1**	Yes	10e8 L	10e7 L
	15.2	No	10e8 L	10e7 L
16	**16.1**	Yes	10e9 D	10e8 D
	16.2	No	10e9 D	10e8D
17	**17.1**	Yes	10e5 D	10e4 D
	17.2	No	10e5 D	10e4 D
18	**18.1**	Yes	10e5 L	10e4 L
	18.2	No	10e5 L	10e4 L
19	**19.1**	Yes	10e6 L 10e6 D	10e5 L 10e5 D
	19.2	No	10e6 L 10e6 D	10e5 L 10e5 D
20	**20.1**	Yes	-	-
	20.2	No	-	-

Controls:

Sample	Cell concentration
C1 L (Live)	10e8
C1 D (Dead)	10e8

9.2 APPENDIX 2 – *TENACIBACULUM MARITIMUM* PROFICIENCY TESTING

Method:
Add 1 mL saline each of the 20 x 100 mg sample in safe lock 2 mL tube with 5 mm stainless steel ball bearing.

Homogenate sample in the Tissue lyser II at 30 Hz for 1 min until sample is homogenous.

Remove 100 µL of the homogeneous solution into duplicate 96-well plates as below.

1.1	2.1	3.1	4.1	5.1	6.1	7.1	8.1	9.1	10.1	11.1	12.1
1.2	2.2	3.2	4.2	5.2	6.2	7.2	8.2	9.2	10.2	11.2	12.2
13.1	14.1	15.1	16.1	17.1	18.1	19.1	20.1				
13.2	14.2	15.2	16.2	17.2	18.2	19.2	20.2				

To one plate; add 2.5 µL PEMAX dye* to X.1 in a BSC in the dark and
mix carefully 3 x by repeat pipetting with a 100 µL pipette. .

Incubate the plate in the dark immediately on a shaking platform at 200 rpm at room temperature (~22 ˚C) for 15 - 30 min.

After incubation, place plate on PAUL and expose to light for 15 min (default setting).

After incubation extract DNA by adding 180 µL ATL buffer and 20 µL proteinase K to each sample, mixing by repeat pipetting and transferring to a screw cap tube.

Incubate at 56 ˚C overnight.

Following lysis, add 100 µL VXL and incubate sample at 65 ˚C for 10 min, remove from
PC3, transfer samples to the lysis block and extract using the QIAcube QIAmp HT kit tissue protocol eluting in 200 µL.

Run all samples in the real time PCR and nested conventional PCR in duplicate.

***PEMAX is a potential carcinogen, use blue gloves when handling it and dispose of waste as cytotoxic.**

Sample	Sample number	Dye	Cell concentration added	Final cell concentration
1	**1.1**	Yes	10e8 L	10e7 L
	1.2	No	10e8 L	10e7 L
2	**2.1**	Yes	10e8 D	10e7 D
	2.2	No	10e8 D	10e7 D
3	**3.1**	Yes	10e7 L 10e5 D	10e6 L 10e4 D
	3.2	No	10e7 L 10e5 D	10e6 L 10e4 D
4	**4.1**	Yes	10e6 D	10e5 D
	4.2	No	10e6 D	10e5 D
5	**5.1**	Yes	10e6 L	10e5 L
	5.2	No	10e6 L	10e5 L
6	**6.1**	Yes	10e5 L	10e4 L
	6.2	No	10e5 L	10e4 L
7	**7.1**	Yes	10e5 D	10e4 D
	7.2	No	10e5 D	10e4 D

8	**8.1**	Yes	10e5 L 10e5 D	10e4 L 10e4 D
	8.2	No	10e5 L 10e5 D	10e4 L 10e4 D
9	**9.1**	Yes	10e4 D	10e3 D
	9.2	No	10e4 D	10e3 D
10	**10.1**	Yes	10e4 L	10e3 L
	10.2	No	10e4 L	10e3 L
11	**11.1**	Yes	10e8 L 10e8 D	10e7 L 10e7 D
	11.2	No	10e8 L 10e8 D	10e7 L 10e7 D
12	**12.1**	Yes	10e6 D	10e5 D
	12.2	No	10e6 D	10e5 D
13	**13.1**	Yes	10e6 L 10e4 D	10e5 L 10e3 D
	13.2	No	10e6 L 10e4 D	10e5 L 10e3 D
14	**14.1**	Yes	10e7 D	10e6 D
	14.2	No	10e7 D	10e6 D
15	**15.1**	Yes	10e7 L	10e6 L
	15.2	No	10e7 L	10e6 L
16	**16.1**	Yes	10e8 D	10e7 D
	16.2	No	10e8D	10e7 D
17	**17.1**	Yes	10e4 D	10e3 D
	17.2	No	10e4 D	10e3 D
18	**18.1**	Yes	10e4 L	10e3 L
	18.2	No	10e4 L	10e3 L
19	**19.1**	Yes	10e6 L 10e6 D	10e5 L 10e5 D
	19.2	No	10e6 L 10e6 D	10e5 L 10e5 D
20	**20.1**	Yes	-	-
	20.2	No	-	-

Controls:
Extract C1-C5 Live and Dead with no treatment

Sample	Cell concentration
C1 L	10e7
C1 D	10e7

9.3 APPENDIX 3 – AQUABIRNAVIRUS PROFICIENCY TESTING

Method:
Add 1 mL saline to the 100 mg sample in tissue lyser tube. Chill the adapter plates prior to use and lyse at 15-30 Hz for 30 sec until the solution is homogeneous.

Remove 100 µL of the homogeneous solution into a 96-well plate in layout as below.

1.1	2.1	3.1	4.1	5.1	6.1	7.1	8.1	9.1	10.1	11.1	12.1
1.2	2.2	3.2	4.2	5.2	6.2	7.2	8.2	9.2	10.2	11.2	12.2
13.1	14.1	15.1	16.1	17.1	18.1	19.1	20.1				
13.2	14.2	15.2	16.2	17.2	18.2	19.2	20.2				

Add 5 µL PEMAX dye* to X.1 in a BSC in the dark. Mix gently by repeat pipetting 3 x and incubate in the dark on ice immediately for 15-20 min.

Place the 96-well plate on PAUL and expose to light at 100 % intensity for 15 min (default setting).

After incubation remove tubes and extract RNA immediately.

Extract RNA by adding 100 µL VXL buffer, 20 µL proteinase K and 1 µL carrier RNA to each sample to each sample, mix carefully and transfer to a screw cap tube. Incubate at 65˚C for 10 minutes. Remove from PC3, transfer samples to the lysis block and extract using the QIAcube cador pathogen kit.

Run all samples in the conventional PCR in duplicate.

***PEMAX is a potential carcinogen, use blue gloves when handling it and dispose of waste as cytotoxic.**

Sample	Sample number	Dye	Cell concentration - initial
1	**1.1**	Yes	Neat – live
	1.2	No	Neat - live
2	**2.1**	Yes	Neat – Dead
	2.2	No	Neat – Dead
3	**3.1**	Yes	Neat live, Neat Dead
	3.2	No	Neat live, Neat Dead
4	**4.1**	Yes	1/10 live, Neat Dead
	4.2	No	1/10 live, Neat Dead
5	**5.1**	Yes	1/10 Dead
	5.2	No	1/10 Dead
6	**6.1**	Yes	1/10 Dead, Neat Live
	6.2	No	1/10 Dead, Neat Live
7	**7.1**	Yes	1/10 live
	7.2	No	1/10 Live
8	**8.1**	Yes	1/10 Live
	8.2	No	1/10 Live
9	**9.1**	Yes	-
	9.2	No	-
10	**10.1**	Yes	1/10 Dead
	10.2	No	1/10 Dead
11	**11.1**	Yes	Neat live, 1/10 Dead
	11.2	No	Neat live, 1/10 Dead
12	**12.1**	Yes	Neat Dead, 1/10 Live
	12.2	No	Neat Dead, 1/10 Live
13	**13.1**	Yes	1/10 Dead
	13.2	No	1/10 Dead
14	**14.1**	Yes	1/10 Live

	14.2	No	1/10 Live
15	**15.1**	Yes	1/10 Dead, 1/10 Live
	15.2	No	1/10 Dead, 1/10 Live
16	**16.1**	Yes	1/100 Live, neat Dead
	16.2	No	1/100 Live, neat Dead
17	**17.1**	Yes	Neat – live
	17.2	No	Neat - live
18	**18.1**	Yes	Neat – Dead
	18.2	No	Neat – Dead
19	**19.1**	Yes	1/10 Dead
	19.2	No	1/10 Dead
20	**20.1**	Yes	1/100 Live
	20.2	No	1/100 Live

Controls:
Extract C1-C5 Live and Dead with no treatment

Sample	Cell concentration
C1 L	Neat
C1D	Neat

9.4 APPENDIX 4 – *YERSINIA RUCKERI*, PHEL FIRST PANEL RESULTS

Average Ct results of the panel sent to PHEL that was processed two days after preparing. These samples gave unexpected results including amplification in the samples not spiked with *Y. ruckeri* (values in red).

Sample	Ct value
10^8 L	17.00
10^8 L	29.35
10^8 D	19.00
10^8 D	26.00
10^7 L 10^5 D	20.42
10^7 L 10^5 D	23.64
10^6 D	29.00
10^6 D	25.00
10^6 L	35.00
10^6 L	27.50
10^5 L	18.00
10^5 L	32.00
10^5 D	22.00
10^5 D	33.50
10^5 L 10^5 D	23.00
10^5 L 10^5 D	27.50
10^4 D	35.50
10^4 D	28.00
10^4 L	26.00
10^4 L	34.00
10^8 L 10^8 D	17.00
10^8 L 10^8 D	24.50
10e6 D	18.50
10e6 D	27.50
10e6 L 10e4 D	20.50
10e6 L 10e4 D	24.00
10e7 D	29.50
10e7 D	25.00
10e7 L	35.00
10e7 L	28.00
10e8 D	18.00
10e8D	25.50
10e4 D	22.00
10e4 D	32.00
10e4 L	18.00
10e4 L	31.00

10e5 L 10e5 D	32.50
10e5 L 10e5 D	28.00
-	26.00
-	36.50

9.5 APPENDIX 5 – STATISTICAL ANALYSIS DATA

9.5.1 Assessment of PEMAX and EMA dye concentration

Yersinia ruckeri

PEMAX:

At a concentration of 10^9 and 10^7 CFU/mL there was a significant difference between the dye concentrations (χ^2 (4, N = 36) = 36.38, p <0.01), (χ^2 (4, N = 36) = 12.373, p <0.01) respectively. The differences were as follows:

Comparison	***p*-value**	
	109 CFU/mL	**107 CFU/mL**
10 – 100 µM	< 0.01*	0.02*
25 - 100 µM	< 0.01*	0.02*
50- 100 µM	0.02*	0.02*
25 – 10 µM	< 0.01*	0.91
50 – 10 µM	< 0.01*	0.91
50 – 25 µM	0.16	0.91

*indicates a significant difference

There was no significant difference between dye concentrations at 10^6 - 10^3 ((χ^2 (4, N = 36) = 5.80, p = 0.12), (χ^2 (4, N = 36) = 1.86, p = 0.60), (χ^2 (4, N = 36) = 0.30, p = 0.96), and (χ^2 (4, N = 36) = 0.49, p = 0.92) respectively).

EMA:

At 10^9 CFU/mL there was a significant difference between the following dye concentrations (χ^2 (4, N = 36) = 77.23, p <0.01). The differences were as follows:

Comparison	***p*-value**
10 – 100 µM	< 0.01*
25 - 100 µM	< 0.01*
50- 100 µM	< 0.01*
25 – 10 µM	< 0.01*
50 – 10 µM	< 0.01*
50 – 25 µM	< 0.01*

*indicates a significant difference

At the concentrations of 10^5 and 10^2 CFU/mL, there was no significant difference between the dye concentrations used (χ^2 (4, N = 36) = 3.29, p = 0.35), (χ^2 (4, N = 36) = 3.82, p = 0.28) respectively.

Comparisons with controls at the bacterial concentration 10^9 CFU mL^{-1} were significant when compared to the controls for both PEMAX and EMA (χ^2 (5, N = 57) = 38.89, p < 0.01) and (χ^2 (5, N = 57) = 23.58, p < 0.01) respectively. The differences were as follows:

Comparison	***p*-value**	
	PEMAX	**EMA**

Dead control + light – Dead control	0.81	0.92
Dead control (no dye) – Dead control	0.99	0.87
Live control – Dead control	0.36	0.09
Dead (treated) – Dead control	<0.1*	0.01*
Dead control (no dye) – Dead control + light	0.81	0.87
Live control – Dead control + light	0.66	0.16
Dead (treated) – Dead control + light	<0.01*	0.13
Live control – Dead control (no dye)	0.46	0.28
Dead (treated) – Dead control (no dye)	<0.01*	0.20
Dead (treated) – Live control	<0.01*	< 0.01*

*indicates a significant difference

Tenacibaculum maritimum

PEMAX:

At a concentration of 10^8 CFU/mL there was a significant difference between dye concentrations (χ^2 (4, N = 36) = 18.304, p <0.01). These differences were as follows:

Comparison	***p*-value**
10 – 100 µM	< 0.01*
25 - 100 µM	0.04*
50- 100 µM	0.60
25 – 10 µM	0.14
50 – 10 µM	< 0.01*
50 – 25 µM	0.09

*indicates a significant difference

At a concentration of 10^5 and 10^3 CFU/mL there was no significant difference between dye concentrations (χ^2 (4, N=36) = 7.37, p = 0.06) and (χ^2 (4, N = 36) = 1.27, p = 0.74).

EMA:

At 10^8 CFU/mL, 10^5 CFU/mL and 10^3 CFU/ml there was no significant difference between the dye concentrations (χ^2 (4, N = 36) = 5.18, p = 0.16), (χ^2 (4, N = 36) = 0.86, p = 0.83) and (χ^2 (4, N = 36) = 0.66, p = 0.88).

Comparisons with controls at the bacterial concentration 10^8 CFU mL^{-1} were significant when compared to the controls for both PEMAX and EMA (χ^2 (5, N = 54) = 30.01, p < 0.01) and (χ^2 (5, N = 54) = 12.87, p = 0.01) respectively. The differences were as follows:

Comparison	***p*-value**	
	PEMAX	**EMA**
Dead control + light – Dead control	0.67	0.79
Dead control (no dye) – Dead control	0.62	0.38
Live control – Dead control	0.96	0.77
Dead (treated) – Dead control	<0.01*	0.16
Dead control (no dye) – Dead control + light	0.98	0.25
Live control – Dead control + light	0.96	0.64

Dead (treated) – Dead control + light	0.02*	0.13
Live control – Dead control (no dye)	0.62	0.64
Dead (treated) – Dead control (no dye)	<0.01*	0.25
Dead (treated) – Live control	0.02*	0.25

*indicates a significant difference

With EMA, there were no differences that were significant when pairwise comparisons were carried out.

ABV

PEMAX:

When analysing the different concentration of dye on a neat concentration of virus, there was a significant difference (χ^2 (4, N = 36) = 10.17, p = 0.02). When further analysing with Tukey contrasts, these differences were not significant:

Comparison	***p*-value**
10 – 100 µM	0.08
25 - 100 µM	0.08
50- 100 µM	0.64
25 – 10 µM	1
50 – 10 µM	0.10
50 – 25 µM	0.10

When analysing the difference at 1/100 and 1/1000 concentrations, there was no significant difference; (χ^2 (4, N = 36) = 7.01, p = 0.07) and (χ^2 (6, N = 36) = 0, p = 1) respectively.

EMA:

When analysing the different dye concentrations, there was no significant difference at neat, 1/100 or 1/1000 viral dilutions; (χ^2 (4, N = 36) = 2.86, p = 0.41), (χ^2 (4, N = 36) = 0.13, p = 0.99), (χ^2 (4, N = 36) = 0, p = 1) respectively.

9.5.2 Assessment of PEMAX and EMA incubation time

Yersinia ruckeri

PEMAX:

At a concentration of 10^9, 10^7, 10^6, 10^5 CFU/mL there was no significant difference between the incubation times (χ^2 (3, N = 36) = 0.07, p = 0.79), (χ^2 (3, N = 36) = 0.07, p = 0.80), (χ^2 (3, N = 36) = 4.74, p = 0.09), (χ^2 (3, N = 36) = 1.25, p = 0.54).

At a concentration of 10^4 and 10^3 CFU/mL, there was a significant difference between the incubation times (χ^2 (3, N = 36) = 8.43, p = 0.01), (χ^2 (3, N = 36) = 9.22, p = 0.01) respectively. The differences were as follows:

Comparison	***p*-value**	
	104 CFU mL^{-1}	**103 CFU mL^{-1}**
20 – 15 mins	0.03*	0.02*
30 - 15 mins	0.03*	0.06

30 – 20 mins	0.79	0.41

EMA:

There was no significant difference between any incubation times at the bacterial concentrations 10^9 or 10^5CFU/mL (χ^2 (3, N = 36) = 0.95, p = 0.62), (χ^2 (3, N = 36) = 2.23, p = 0.33) respectively.

Tenacibaculum maritimum

PEMAX:

When analysing the incubation times for PEMAX, there was no significant difference on any bacterial concentration 10^8 CFU/mL, 10^5 CFU/mL and 10^3 CFU/ml; (χ^2 (3, N = 36) = 0.3556, p = 0.55), (χ^2 (3, N = 36) = 0.24, p = 0.62), and (χ^2 (3, N = 36) = 0.86, p = 0.35) respectively.

EMA:

When analysing the incubation times for each bacterial concentration there was no significant difference between any time at 10^8, 10^5 or 10^3 CFU/ml (χ^2 (3, N = 36) = 0.57, p = 0.75), (χ^2 (3, N = 36) = 0.02, p = 0.99), (χ^2 (3, N = 36) = 2.54, p = 0.28) respectively.

ABV

PEMAX:

There was no significant difference on incubation time using neat, 1/100 or 1/1000 viral concentrations; (χ^2 (3, N = 36) = 0.69, p = 0.71), 1/100 (χ^2 (3, N = 36) = 0, p = 1) or 1/1000 (χ^2 (3, N = 36) = 0, p = 1) respectively.

EMA:

There was no significant difference between any incubation time with neat, 1/100 or 1/1000 viral dilutions (χ^2 (3, N = 36) = 2.25, p = 0.32), (χ^2 (3, N = 36) = 0, p = 1), (χ^2 (3, N = 36) = 0, p = 1) respectively.

9.5.3 Assessment on incubation temperature with EMA and PEMAX dye

Yersinia ruckeri

PEMAX:

Analysis was carried out on dead cells only (not ratios) at both 10^9 and 10^5 CFU/mL cell concentrations. There was a significant difference between temperatures at 10^9 CFU/mL (χ^2 (5, N = 12) = 11.62, p <0.01) but not at 10^5 CFU/mL (χ^2 (5, N = 12) = 1.01, p = 0.80). The differences for 10^9 CFU/mL were as follows:

Comparison	***p*-value**
4 – 0 °C	< 0.01*
30 – 0 °C	0.02*
30 – 22 °C	0.57
4 – 22 °C	0.37
4 – 30 °C	0.57
22 – 0 °C	0.05*

*indicates a significant difference

EMA:

No significant difference was seen between temperatures of dead cells at 10^9 or 10^5 CFU/mL (χ^2 (5, N = 12) = 2.35, p = 0.50), (χ^2 (5, N = 12) = 6.23, p = 0.10) respectively.

Analysing all groups (live, dead and controls) for PEMAX and EMA showed significant differences (χ^2 (5, N = 60) = 19.83, p < 0.01) and (χ^2 (5, N = 60) = 19.13, p < 0.01) respectively. The difference were as follows:

Comparison	***p*-value**	
	PEMAX	**EMA**
Dead_control – Dead	0.06	0.06
Live - Dead	< 0.01*	< 0.01*
Live_control - Dead	0.02*	0.02*
Live – Dead_control	0.77	0.76
Live_control – Dead_control	0.77	0.76
Live_control - Live	0.98	0.97

*indicates a significant difference

Tenacibaculum maritimum

PEMAX:

Statistical analysis carried out on dead cells at 10^8, 10^5 and 10^3 CFU/mL showed there was no significant difference between temperatures at any bacterial concentration; (χ^2 (5, N = 12) = 6.33, p = 0.10), (χ^2 (5, N = 12) = 3.07, p = 0.38) (χ^2 (5, N=12) = 0.41, p = 0.94) respectively.

When analysing live cells, there was a significant difference between temperatures at 10^8 CFU/mL (χ^2 (5, N = 12) = 9.31, p = 0.03). The differences were as follows:

Comparison	***p*-value**
22 – 0 °C	0.06
30 – 0 °C	0.34
4 – 0 °C	0.03*
30 – 22 °C	0.33
4 – 22 °C	0.66
4 – 30 °C	0.18

*indicates a significant difference

There was no significant difference between temperatures at 10^5 or 10^3 CFU/mL; (χ^2 (5, N =12) = 2.40, p = 0.49) (χ^2 (5, N = 12) = 0.86, p = 0.84) respectively.

When analysing the difference between dead, control and live at 10^8, 10^5 and 10^3 CFU/mL, there was a significant difference (χ^2 (4, N = 30) = 57.66, p < 0.01), (χ^2 (4, N = 30) = 18.12, p < 0.01), (χ^2 (4, N = 30) = 9.63, p = 0.02) respectively. The differences were as follows:

Comparison	***p*-value**		
	10^8 CFU mL^{-1}	**10^5 CFU mL^{-1}**	**10^3 CFU mL^{-1}**
Dead_control – Dead	< 0.01*	0.01*	0.33
Live - Dead	< 0.01*	0.06	0.04*
Live_control - Dead	< 0.01*	< 0.01*	0.04*

Live – Dead_control	0.08	0.16	0.70
Live_control – Dead_control	0.02*	0.28	0.33
Live_control - Live	0.18	< 0.01*	0.33

*indicates a significant difference

EMA:

Analysis was carried out on dead cells at 10^8, 10^5 and 10^3 CFU/mL and found there was no significant difference between temperatures at each bacterial concentration (χ^2 (4, N = 12) = 8.46, p = 0.04), (χ^2 (4, N = 12) = 6.13, p =0.11), (χ^2 (4, N = 12) = 1.67, p =0.64) respectively.

When analysing the live cells to see if there was any differences between the temperatures it revealed there was no significant difference at 10^8 or 10^3 CFU/mL (χ^2 (4, N = 12) = 3.37, p = 0.34), (χ^2 (4, N = 12) = 0.20, p = 0.98) respectively.

At 10^5 CFU/mL there was a significant difference (χ^2 (4, N = 12) = 24.39, p < 0.01). The differences were as follows:

Comparison	***p*-value**
30 – 22 °C	0.03*
4 – 22 °C	< 0.01*
0 – 22 °C	0.45
4 – 30 °C	< 0.01*
0 – 30 °C	< 0.01*
0 – 4 °C	< 0.01*

*indicates a significant difference

When analysing the difference between dead, control and live cells at 10^8, 10^5, 10^3 CFU/mL, there was a significant difference (χ^2 (4, N = 22) = 33.951, p < 0.01), (χ^2 (4, N = 22) = 12.69, p = 0.01), (χ^2 (4, N = 22) = 17.80, p = 0.01) respectively. The differences were as follows:

Comparison	***p*-value**		
	10^8 CFU mL^{-1}	**10^5 CFU mL^{-1}**	**10^3 CFU mL^{-1}**
Dead_control – Dead	0.01*	0.08	0.34
Live - Dead	< 0.01*	0.19	0.89
Live_control - Dead	< 0.01*	< 0.01*	< 0.01*
Live – Dead_control	0.01*	0.3	0.34
Live_control – Dead_control	0.04*	0.24	0.01*
Live_control - Live	0.85	0.04*	< 0.01*

*indicates a significant difference

ABV

EMA:

Statistical analysis carried out on dead cells at a neat viral concentration showed a significant difference between the temperatures (χ^2 (4, N = 12) = 12.48, p = 0.01). However, when the multiple comparison of means was carried out, there was no significance between any of the groups (p = 1). At viral concentrations of 1/10 and 1/100 there was no significant difference between the temperatures (both χ^2 (4, N = 12) = 0, p = 1).

When analysing live virus, there was no significant difference between temperatures at neat, 1/100 and 1/1000 (χ^2 (4, N = 12) = 0, p = 1), (χ^2 (4, N = 12) = 3.01, p = 0.38) (χ^2 (4, N = 12) = 0, p = 1) respectively.

When analysing live, dead and controls, there was a significant difference (χ^2 (5, N = 90) = 48.62, p < 0.01). The differences were as follows:

Comparison	*p*-value
Dead_control – Dead	1
Live - Dead	< 0.01*
Live_control - Dead	1
Live – Dead_control	1
Live_control – Dead_control	1
Live_control - Live	1

*indicates a significant difference

PEMAX:

When analysing dead cells, at a neat, 1/100 and 1/1000 viral concentration there was no significant difference between temperatures (χ^2 (4, N = 12) = 0, p = 1).

When analysing live virus, there was no significant difference between temperatures at neat, 1/100 and 1/1000 (χ^2 (4, N = 12) = 0, p = 1), (χ^2 (4, N = 12) = 0, p = 1) (χ^2 (4, N = 12) = 3.07, p = 0.38) respectively.

When analysing live, dead and controls, there was a significant difference (χ^2 (4, N = 27) = 70.20, p < 0.01). However, when the multiple comparison of means was carried out, there was no significance between any of the groups (p = 0.996).

9.5.4 Assessment of PhAST photoperiod

Yersinia ruckeri

PEMAX:

When analysing dead cells at 10^9 CFU/mL, there was no significant difference between any exposure time (χ^2 (4, N = 12) = 3.58, p = 0.31).

When analysing dead cells at 10^5 CFU/mL there was a significant difference between the exposure times (χ^2 (4, N = 15) = 8.12, p = 0.04). However, when the multiple comparison of means was carried out, there was no significance between any of the groups:

Comparison	*p*-value
15 – 10 mins	0.11
30 – 10 mins	0.90
5 – 10 mins	0.90
30 – 15 mins	0.11
5 – 15 mins	0.11
5 – 30 mins	0.90

When analysing live cells, there was no significant difference with between the exposure times at both bacterial concentrations; 10^9 and 10^5 CFU/mL (χ^2 (4, N = 12) = 7.54, p = 0.06), (χ^2 (4, N = 12) = 1.19, p = 0.76) respectively.

When analysing live, dead and controls there was a significant difference (χ^2 (4, N = 66) = 19.98, p < 0.01). The differences were as follows:

Comparison	*p*-value
Dead_control – Dead	0.03*
Live - Dead	< 0.01*
Live_control - Dead	0.02*
Live – Dead_control	0.93
Live_control – Dead_control	0.93
Live_control - Live	0.93

*indicates a significant difference

EMA:

Statistical analysis of dead cells at 10^9 CFU/mL, there was no significant difference between any exposure time (χ^2 (4, N = 48) = 0.07, p = 0.58).
Statistical analysis of dead cells at 10^5 CFU/mL, showed a significant difference (χ^2 (4, N = 48) = 11.59, p = 0.01). The differences were as follows:

Comparison	*p*-value
15 – 10 mins	0.02*
30 – 10 mins	0.03*
5 – 10 mins	0.02*
30 – 15 mins	0.90
5 – 15 mins	0.90
5 – 30 mins	0.90

*indicates a significant difference

When analysing live cells, there was no significant difference with between the exposure times at both concentrations (χ^2 (4, N = 48) = 0.04, p = 0.99).

When analysing live, dead and controls there was a significant difference (χ^2 (6, N = 108) = 38.00, p < 0.01). The differences were as follows:

Comparison	*p*-value
Dead_control – Dead	0.01*
Live - Dead	0.01*
Live_control - Dead	< 0.01*
Live – Dead_control	0.85
Live_control – Dead_control	0.85
Live_control - Live	0.85

*indicates a significant difference

Tenacibaculum maritimum

PEMAX:

Statistical analysis of dead cells at 10^8 and 10^3 CFU/mL, showed there was a significant difference between the exposure times (χ^2 (4, N =12) = 10.37, p = 0.02), (χ^2 (4, N =12) = 8.37, p = 0.04) respectively. The differences were as follows:

Comparison	*p*-value	
	10^8 CFU mL^{-1}	10^3 CFU mL^{-1}

15 – 10 mins	0.01*	0.44
30 – 10 mins	0.46	0.03*
5 – 10 mins	0.46	0.27
30 – 15 mins	0.05*	0.19
5 – 15 mins	0.05*	0.56
5 – 30 mins	0.94	0.30

*indicates a significant difference

At dead cell concentrations of 105 CFU/mL, there was no significant difference between the exposure times ($\chi 2$ (4, N =12) = 5.21, p = 0.16).

Statistical analysis of live cells at the different exposure times at bacterial concentrations of 108 and 103 CFU/mL showed no significant difference between exposure times ($\chi 2$ (4, N =12) = 4.46, p = 0.22), ($\chi 2$ (4, N =12) = 6.72, p = 0.08) respectively.
At live cell concentrations of 105 CFU/mL there was a significant difference ($\chi 2$ (4, N =12) = 13.20, p < 0.01). The differences were as follows:

Comparison	***p*-value**
15 – 10 mins	< 0.01*
30 – 10 mins	0.05*
5 – 10 mins	< 0.01*
30 – 15 mins	0.17
5 – 15 mins	0.47
5 – 30 mins	0.47

*indicates a significant difference

When analysing live, dead and controls there was a significant difference (χ^2 (4, N = 90) = 9.15, p = 0.03). A likelihood ratio test was carried out which showed that none of the differences were significant between the treatment groups. When analysing further and looking at the separate bacterial concentrations, 10^8,10^5 and 10^3 CFU/mL showed a significant difference (χ^2 (4, N = 30) = 94.76, p = < 0.01), (χ^2 (4, N = 30) = 48.66, p = < 0.01), (χ^2 (4, N = 30) = 17.12, p = < 0.01).

Comparison	***p*-value**			
	All	10^8 CFU mL^{-1}	10^5 CFU mL^{-1}	10^3 CFU mL^{-1}
Dead_control – Dead	0.15	< 0.01*	< 0.01*	0.04*
Live - Dead	0.07	< 0.01*	0.64	0.61
Live_control - Dead	0.07	< 0.01*	< 0.01*	< 0.01*
Live – Dead_control	0.73	< 0.01*	< 0.01*	0.02*
Live_control – Dead_control	0.72	0.06	< 0.01*	0.37
Live_control - Live	0.47	0.56	< 0.01*	< 0.01*

*indicates a significant difference

EMA:

Statistical analysis of the dead cells at 10^8 CFU/mL showed there was a significant difference between the exposure times (χ^2 (4, N =12) = 13.27, p < 0.01). The differences were as follows:

Comparison	***p*-value**
15 – 10 mins	0.60
30 – 10 mins	0.10

5 – 10 mins	0.05*
30 – 15 mins	0.05*
5 – 15 mins	0.10
5 – 30 mins	< 0.01*

*indicates a significant difference

At bacterial concentrations of 10^5 and 10^3 CFU/mL, there was no significant difference in the dead cells between any exposure times (χ^2 (4, N =12) = 3.74, p = 0.29), (χ^2 (4, N =12) = 5.32, p = 0.15) respectively.

When analysing live cells, there was no significant difference with between the exposure times at 10^8 or 10^5 CFU/mL (χ^2 (4, N = 12) = 3.14, p = 0.37), (χ^2 (4, N = 12) = 4.28, p = 0.23) respectively. At 10^3 CFU/mL, there was a significant difference (χ^2 (4, N = 12) = 9.04, p = 0.03).The differences were as follows:

Comparison	***p*-value**
15 – 10 mins	0.20
30 – 10 mins	0.20
5 – 10 mins	0.86
30 – 15 mins	0.02*
5 – 15 mins	0.20
5 – 30 mins	0.20

*indicates a significant difference

When analysing live, dead and controls there was a significant difference (χ^2 (6, N = 108) = 12.76, p < 0.01). The differences were as follows:

Comparison	***p*-value**
Dead_control – Dead	0.04*
Live - Dead	0.04 *
Live_control - Dead	0.02*
Live – Dead_control	0.48
Live_control – Dead_control	0.60
Live_control - Live	0.20

*indicates a significant difference

9.5.5 Assessing the use of resuspension buffers

Yersinia ruckeri

PEMAX and EMA:

Statistical analysis on the dead cells showed there was a significant difference between the buffers used with PEMAX (χ^2 (15, N = 42) = 38.056, p < 0.01). No significant difference was seen between any buffers for EMA (χ^2 (15, N = 42) = 14.0, p = 0.37). When analysing the live cells for PEMAX and EMA, there was no significant difference between the buffers for PEMAX (χ^2 (15, N = 42) = 17.03, p = 0.20) but there was a significant difference for EMA (χ^2 (15, N = 42) = 32.91, p < 0.01).

The differences for PEMAX dead and EMA live are as follows:

Comparison	*p*-value	
	PEMAX dead	**EMA live**
0.3 % SD – 0.01 % SD	0.38	0.98
0.1 % SD – 0.01 % SD	0.84	0.83
0.1% Triton X – 0.01 % SD	0.70	0.90
0.3 % SD – 0.01 % SD	0.04*	0.95
0.5 % Triton X – 0.01 % SD	0.82	0.83
1 % Triton X – 0.01 % SD	0.82	0.25
Broth – 0.01 % SD	< 0.01*	< 0.01*
PBS – 0.01 % SD	0.39	0.86
pH 7 – 0.01 % SD	0.82	0.90
pH 7.5 – 0.01 % SD	0.70	0.90
pH 8 – 0.01 % SD	0.86	0.95
pH 8.5 – 0.01 % SD	0.52	0.90
Reaction buffer – 0.01 % SD	0.87	0.94
0.1 % SD – 0.03 % SD	0.52	0.83
0.1 % Triton X – 0.03 % SD	0.12	0.93
0.3 % SD – 0.03 % SD	0.44	0.95
0.5 % Triton X – 0.03 % SD	0.44	0.83
1 % Triton X – 0.03 % SD	0.24	0.23
Broth – 0.03 % SD	0.12	< 0.01*
PBS – 0.03 % SD	0.98	0.83
pH 7 – 0.03 % SD	0.52	0.90
pH 7.5 – 0.03 % SD	0.70	0.90
pH 8 – 0.03 % SD	0.52	0.95
pH 8.5 – 0.03 % SD	0.86	0.90
Reaction buffer – 0.03 % SD	0.28	0.94
0.1 % Triton X – 0.1 % SD	0.52	0.48
0.3 % SD – 0.1 % SD	0.08	0.90
0.5 % Triton X – 0.1 g SD	0.70	1
1 % Triton X – 0.1 % SD	0.70	0.83
Broth – 0.1 % SD	0.01*	< 0.01*
PBS – 0.1 % SD	0.52	0.98
pH 7 – 0.1 % SD	0.98	0.95
pH 7.5 – 0.1 % SD	0.82	0.95
pH 8 – 0.1 % SD	0.98	0.90
pH 8.5 – 0.1 % SD	0.67	0.95
Reaction buffer – 0.1 % SD	0.75	0.94
0.3 % SD – 0.1 % Triton X	0.01*	0.83
0.5 % Triton X – 0.1 % Triton X	0.82	0.48
1 % Triton X – 0.1 % Triton X	0.82	0.05*

Broth – 0.1 % Triton X	< 0.01*	< 0.01*
PBS – 0.1 % Triton X	0.12	0.50
pH 7 – 0.1 % Triton X	0.52	0.67
pH 7.5 – 0.1 % Triton X	0.34	0.63
pH 8 – 0.1 % Triton X	0.52	0.88
pH 8.5 – 0.1 % Triton X	0.20	0.63
Reaction buffer – 0.1 % Triton X	0.78	0.82
0.5 % Triton X – 0.3 % SD	0.01*	0.90
1 % Triton X – 0.3 % SD	0.01*	0.42
Broth – 0.3 % SD	0.63	< 0.01*
PBS – 0.3 % SD	0.43	0.93
pH 7 – 0.3 % SD	0.08	0.95
pH 7.5 – 0.3 % SD	0.17	0.94
pH 8 – 0.3 % SD	0.07	0.98
pH 8.5 – 0.3 % SD	0.32	0.94
Reaction buffer – 0.3 % SD	0.02	0.97
1 % Triton X – 0.5 % Triton X	0.98	0.83
Broth – 0.5 % Triton X	< 0.01*	< 0.01*
PBS – 0.5 % Triton X	0.24	0.98
pH 7 – 0.5 % Triton X	0.70	0.95
pH 7.5 – 0.5 % Triton X	0.52	0.95
pH 8 – 0.5 % Triton X	0.71	0.90
pH 8.5 – 0.5 % Triton X	0.34	0.95
Reaction buffer – 0.5 % Triton X	0.96	0.94
Broth – 1 % Triton X	< 0.01*	0.09
PBS – 1 % Triton X	0.24	0.82
pH 7 – 1 % Triton X	0.70	0.63
pH 7.5 – 1 % Triton X	0.52	0.67
pH 8 – 1 % Triton X	0.70	0.38
pH 8.5 – 1 % Triton X	0.34	0.67
Reaction buffer – 1 % Triton X	0.95	0.49
PBS – Broth	0.12	< 0.01*
pH 7 – Broth	0.01*	< 0.01*
pH 7.5 – Broth	0.02*	< 0.01*
pH 8 – Broth	0.01*	< 0.01*
pH 8.5 – Broth	0.07	< 0.01*
Reaction buffer – Broth	< 0.01*	< 0.01*
pH 7 – PBS	0.52	0.95
pH 7.5 – PBS	0.70	0.97
pH 8 – PBS	0.52	0.90
pH 8.5 – PBS	0.87	0.97

Reaction buffer – PBS	0.89	0.94
pH 7.5 – pH 7	0.82	0.97
pH 8 – pH 7	0.96	0.94
pH 8.5 – pH 7	0.69	0.98
Reaction buffer – pH 7	0.73	0.95
pH 8 – pH 7.5	0.82	0.94
pH 8.5 – pH 7.5	0.82	0.99
Reaction buffer – pH 7.5	0.56	0.95
pH 8.5 – pH 8	0.78	0.94
Reaction buffer – pH 8	0.78	0.95
Reaction buffer – pH 9.5	0.39	0.95

*indicates a significant difference

When analysing live cells, there was no significant difference between the buffers (χ^2 (15, N = 42) = 17.03, p = 0.20).

When analysing the live, dead and controls, there was a significant difference with PEMAX and EMA (χ^2 (, N = 90) = 148.64, p < 0.01), (χ^2 (, N = 90) = 237.09, p < 0.01) respectively. The differences were as follows:

Comparison	***p*-value**	
	PEMAX	**EMA**
Dead_control – Dead	< 0.01*	< 0.01*
Live - Dead	< 0.01*	< 0.01*
Live_control - Dead	< 0.01*	< 0.01*
Live – Dead_control	0.40	0.15
Live_control – Dead_control	0.40	0.16
Live_control - Live	0.77	0.63

*indicates a significant difference

Tenacibaculum maritimum

PEMAX and EMA:

Statistical analysis on the dead cells showed there was a significant difference between the buffers used with PEMAX and EMA (χ^2 (15, N = 48) = 35.14, p < 0.01), (χ^2 (15, N = 48) = 38.16, p < 0.01*).
When analysing the live cells for PEMAX and EMA, there was no significant difference between the buffers for PEMAX or EMA (χ^2 (15, N = 48) = 15.39, p = 0.43), (χ^2 (15, N = 48) = 16.32, p = 36) respectively.

The differences for PEMAX and EMA dead are as follows:

Comparison	***p*-value**	
	PEMAX dead	**EMA dead**
0.3 % SD – 0.01 % SD	0.99	0.82
0.1 % SD – 0.01 % SD	0.96	0.39
0.1 % Triton X – 0.01 % SD	0.99	0.89
0.3 % SD – 0.01 % SD	0.99	0.69
0.5 % Triton X – 0.01 % SD	0.99	0.86

1 % Triton X – 0.01 % SD	0.99	0.77
Broth – 0.01 % SD	0.04*	0.16
PBS – 0.01 % SD	0.99	0.9
pH 7 – 0.01 % SD	0.99	0.05*
pH 7.5 – 0.01 % SD	0.99	0.91
pH 8 – 0.01 % SD	0.99	0.81
pH 8.5 – 0.01 % SD	0.99	0.89
Reaction buffer – 0.01 % SD	0.99	0.92
Saline – 0.1 % SD	0.99	0.75
Seawater – 0.1 % SD	0.02*	0.4
0.1 % SD – 0.03 % SD	0.87	0.65
0.1 % Triton X – 0.03 % SD	0.99	0.75
0.3 % SD – 0.03 % SD	0.99	0.86
0.5 % Triton X – 0.03 g SD	0.99	0.94
1 % Triton X – 0.03 % SD	0.99	0.90
Broth – 0.03 % SD	0.03*	0.34
PBS – 0.03 % SD	0.99	0.85
pH 7 – 0.03 % SD	0.99	0.11
pH 7.5 – 0.03 % SD	0.89	0.89
pH 8 – 0.03 % SD	0.99	0.64
pH 8.5 – 0.03 % SD	0.99	0.75
Reaction buffer – 0.03 % SD	0.99	0.89
Saline – 0.03 % SD	0.99	0.47
Seawater – 0.03 % SD	0.02*	0.08
0.1 % Triton X – 0.1 % SD	0.82	0.26
0.3 % SD – 0.1 % SD	0.99	0.81
0.5 % Triton X – 0.1 % SD	0.82	0.60
1 % Triton X – 0.1 % SD	0.46	0.75
Broth – 0.1 % SD	0.27	0.81
PBS – 0.1 % SD	0.87	0.41
pH 7 – 0.1 % SD	0.99	0.49
pH 7.5 – 0.1 % SD	0.99	0.50
pH 8 – 0.1 % SD	0.99	0.17
pH 8.5 – 0.1 % SD	0.84	0.26
Reaction buffer – 0.1 % SD	0.80	0.47
Saline – 0.1 % SD	0.84	0.11
Seawater – 0.1 % SD	0.15	0.39
0.3 % SD – 0.1 % Triton X	0.99	0.52
0.5 % Triton X – 0.1 % Triton X	0.99	0.77
1 % Triton X – 0.1 % Triton X	0.99	0.64
Broth – 0.1 % Triton X	0.02*	0.09

PBS – 0.1 % Triton X	0.99	0.89
pH 7 – 0.1 % Triton X	0.99	0.03*
pH 7.5 – 0.1 % Triton X	0.83	0.81
pH 8 – 0.1 % Triton X	0.99	0.89
pH 8.5 – 0.1 % Triton X	0.99	0.99
Reaction buffer – 0.1 % Triton X	0.99	0.85
Saline – 0.1 % Triton X	0.99	0.81
Seawater – 0.1 % Triton X	0.02*	0.02*
0.5 % Triton X – 0.3 % SD	0.99	0.81
1 % Triton X – 0.3 % SD	0.99	0.91
Broth – 0.3 % SD	0.04*	0.52
PBS – 0.3 % SD	0.99	0.71
pH 7 – 0.3 % SD	0.99	0.22
pH 7.5 – 0.3 % SD	0.99	0.77
pH 8 – 0.3 % SD	0.99	0.41
pH 8.5 – 0.3 % SD	0.99	0.52
Reaction buffer – 0.3 % SD	0.99	0.75
Saline – 0.3 % SD	0.99	0.27
Seawater – 0.03 % SD	0.02*	0.16
1% Triton X – 0.5 % Triton X	0.99	0.89
Broth – 0.5 % Triton X	0.02*	0.28
PBS – 0.5 % Triton X	0.99	0.89
pH 7 – 0.5 % Triton X	0.99	0.09
pH 7.5 – 0.5 % Triton X	0.82	0.91
pH 8 – 0.5 % Triton X	0.99	0.68
pH 8.5 – 0.5 % Triton X	0.99	0.77
Reaction buffer – 0.5 % Triton X	0.99	0.89
Saline – 0.5 % Triton X	0.99	0.52
Seawater – 0.5 % Triton X	0.02*	0.07
Broth – 1 % Triton X	0.02*	0.45
PBS – 1 % Triton X	0.99	0.77
pH 7 – 1 % Triton X	0.89	0.16
pH 7.5 – 1 % Triton X	0.48	0.81
pH 8 – 1 % Triton X	0.87	0.50
pH 8.5 – 1 % Triton X	0.99	0.64
Reaction buffer – 1 % Triton X	0.99	0.81
Saline – 1 % Triton X	0.99	0.37
Seawater – 1 % Triton X	0.02*	0.11
PBS – Broth	0.03*	0.16
pH 7 – Broth	0.07	0.77
pH 7.5 – Broth	0.07	0.22

pH 8 – Broth	0.02*	0.06
pH 8.5 – Broth	0.02*	0.09
Reaction buffer – Broth	0.02*	0.19
Saline – Broth	0.02*	0.04*
Seawater – Broth	0.99	0.71
pH 7 – PBS	0.99	0.05*
pH 7.5 – PBS	0.87	0.92
pH 8 – PBS	0.99	0.81
pH 8.5 – PBS	0.99	0.89
Reaction buffer – PBS	0.99	0.95
Saline – PBS	0.99	0.71
Seawater – PBS	0.02*	0.04*
pH 7.5 – pH 7	0.99	0.07
pH 8 – pH 7	0.99	0.02*
pH 8.5 – pH 7	0.99	0.03*
Reaction buffer – pH 7	0.99	0.06
Saline – pH 7	0.99	0.02*
Seawater – pH 7	0.04*	0.91
pH 8 – pH 7.5	0.99	0.76
pH 8.5 – pH 7.5	0.85	0.81
Reaction buffer – pH 7.5	0.82	0.96
Saline – pH 7.5	0.99	0.64
Seawater – pH 7.5	0.14	0.05*
pH 8.5 – pH 8	0.99	0.89
Reaction buffer – pH 8	0.99	0.77
Saline – pH 8	0.99	0.89
Seawater – pH 8	0.04*	0.02*
Reaction buffer – pH 8.5	0.99	0.85
Saline – pH 8.5	0.99	0.81
Seawater – pH 8.5	0.02*	0.02*
Saline – Reaction buffer	0.99	0.66
Seawater – Reaction buffer	0.02*	0.05*
Seawater – saline	0.02*	0.02*

*indicates a significant difference

When analysing the live, dead and controls, there was a significant difference with PEMAX and EMA (χ^2 (4, N = 102) = 150.9, $p < 0.01$), (χ^2 (4, N = 102) = 171.56, $p < 0.01$) respectively. The differences were as follows:

Comparison	***p*-value**	
	PEMAX	**EMA**
Dead_control – Dead	< 0.01*	< 0.01*
Live - Dead	< 0.01*	< 0.01*

Live_control - Dead	< 0.01*	< 0.01*
Live – Dead_control	0.35	0.43
Live_control – Dead_control	0.35	0.36
Live_control - Live	0.79	0.48

*indicates a significant difference

9.5.6 Assessment of double dye method

Yersinia ruckeri

Analysis of the Ct value in the dead cells between all dye exposures of PEMAX, there was a significant difference (χ^2 (4, N=9) = 29.01, p < 0.01). The differences were as follows:

Comparison	***p*-value**
2 x 100 – 1 x 100	< 0.01*
2 x 50 – 1 x 100	< 0.01*
2 x 50 – 2 x 100	< 0.01*

*indicates a significant difference

No significant differences were seen between the exposures on the live cells (p > 0.05).

When comparing the live, dead and controls for both PEMAX and EMA differences were significant (χ^2 (4, N=24) = 74.83, p < 0.01) and (χ^2 (4, N=24) = 64.10, p < 0.01) respectively. The differences were as follows:

Comparison	***p*-value**	
	PEMAX	**EMA**
Dead_control – Dead	< 0.01*	< 0.01*
Live - Dead	< 0.01*	< 0.01*
Live_control - Dead	< 0.01*	< 0.01*
Live – Dead_control	0.81	0.44
Live_control – Dead_control	0.20	0.80
Live_control - Live	0.08	0.34

*indicates a significant difference

No differences were seen between the Ct values of the dead or live cells exposed to the difference amounts of EMA (χ^2 (4, N=9) = 3.59, p = 0.17) and (χ^2 (4, N=9) = 3.21, p = 0.20) respectively.

Tenacibaculum maritimum

Analysis of the Ct value in the dead cells between single and double PEMAX dye exposures showed a significant difference (χ^2 (2, N=6) = 8.5, p < 0.01).

Analysis of the Ct values in the live cells showed a significant difference (χ^2 (2, N=6) = 7.97, p < 0.01).

Analysis of the Ct values in the dead and live cells between single and double EMA dye exposures showed no significant difference (χ^2 (2, N=6) = 0.081, p = 0.78) and (χ^2 (2, N=6) = 2.13, p = 0.15) respectively.

When comparing the live, dead and controls for both PEMAX and EMA differences were significant (χ^2 (4, N=18) = 34.47, p < 0.01) and (χ^2 (4, N=18) = 56.28, p < 0.01) respectively. The differences were as follows:

Comparison	*p*-value	
	PEMAX	**EMA**
Dead_control – Dead	< 0.01*	< 0.01*
Live - Dead	< 0.01*	< 0.01*
Live_control - Dead	< 0.01*	< 0.01*
Live – Dead_control	0.39	< 0.01*
Live_control – Dead_control	0.07	< 0.01*
Live_control - Live	0.19	0.74

*indicates a significant difference

9.5.7 Assessment of longer incubation time with PEMAX for *Yersinia ruckeri*

No significant difference was seen on dead cells between the incubation times (χ^2 (5, N=15) = 6.51, p = 0.16). When looking at live cells there was a significant difference (χ^2 (5, N=15) = 22.91, p < 0.01). The differences were as follows:

Comparison	*p*-value
45-30 min	0.71
60-30 min	< 0.01*
75-30 min	0.01*
90-30 min	< 0.01*
60-45 min	< 0.01*
75-45 min	< 0.01*
90-45 min	< 0.01*
75-60 min	0.54
90-60 min	0.66
90-75 min	0.27

*indicates a significant difference

Analysing all the groups there was a significant difference (χ^2 (4, N=36) = 138.85, p < 0.01). These differences were as follows:

Comparison	*p*-value
Dead_control - Dead	< 0.01*
Live - Dead	< 0.01*
Live_control – Dead	< 0.01*
Live – Dead_control	0.02*
Live_control – Dead_control	0.55
Live_control - Live	0.09

*indicates a significant difference

9.5.8 Assessment of "double tube" method

***Yersinia ruckeri* - 10^9 CFU mL^{-1}**

When analysing the difference of the Ct value in the dead cells between all the treatments, there was a significant difference (χ^2 (4, N=15) = 70.82, $p < 0.01$). The differences were as follows:

Comparison	*p*-value
2 tube (1st tube) – single tube	< 0.01*
Dark tube (1st) – single tube	< 0.01*
2 tube (2nd) – 2 tube (1st)	< 0.01*
Dark tube (1st) – 2 tube (1st)	< 0.01*
Dark tube (2nd) – 2 tube (1st)	< 0.01*
Dark tube (1st) – 2 tube (2nd)	< 0.01*
Dark tube (2nd) – dark tube (1st)	< 0.01*
2 tube (2nd) – single tube	0.63
Dark tube (2nd) – single tube	0.63
Dark tube (2nd) – 2 tube (2nd)	0.38

*indicates a significant difference

As the 1st tube of the two tube methods was just DNA debris, analysis was carried out excluding these results. When looking at these samples, there was no significant difference between using a single tube or two tubes, or using a light sensitive tube (dark) (χ^2 (3, N=9) = 2.323, p = 0.31).

When analysing the difference in the Ct value of the live cells between all treatments, there was a significant difference (χ^2 (4, N=15) = 71.29, $p < 0.01$). The difference were as follows:

Comparison	*p*-value
2 tube (1st) – single tube	< 0.01*
2 tube (2nd) – single tube	0.25
Dark tube (1st) – single tube	< 0.01*
Dark tube (2nd) – single tube	0.32
2 tube (2nd) – 2 tube (1st)	< 0.01*
Dark tube (1st) – 2 tube (1st)	0.13
Dark tube (2nd) – 2 tube (1st)	< 0.01*
Dark tube (1st) – 2 tube (2nd)	< 0.01*
Dark tube (2nd) – 2 tube (2nd)	0.82
Dark tube (2nd) – Dark tube (1st)	< 0.01*

*indicates a significant difference

When just looking at the 2nd tube of the two tube method and comparing that to the single tube method, there was no significant difference between using a single tube or two tubes, or using a light sensitive tube (dark) (χ2 (3, N=9) = 3.17, p = 0.21).

***Yersinia ruckeri* – 10^5 CFU mL^{-1}**

When analysing the difference of the Ct value in the dead cells between all the treatments, there was a significant difference (χ2 (4, N=15) = 26.48, p < 0.01). These differences were as follows:

Comparison	*p*-value
2 tube (1st tube) – single tube	0.37
Dark tube (1st) – single tube	< 0.01*
2 tube (2nd) – 2 tube (1st)	0.74
Dark tube (1st) – 2 tube (1st)	< 0.01*
Dark tube (2nd) – 2 tube (1st)	< 0.01*
Dark tube (1st) – 2 tube (2nd)	< 0.01*
Dark tube (2nd) – dark tube (1st)	0.94
2 tube (2nd) – single tube	0.60
Dark tube (2nd) – single tube	< 0.01*
Dark tube (2nd) – 2 tube (2nd)	< 0.01*

*indicates a significant difference

As the 1st tube of the two tube methods was just DNA debris, analysis was carried out excluding these results. When looking at these samples, there was a significant difference between using a single tube or two tubes, or using a light sensitive tube (dark) (χ^2 (3, N=9) = 11.39, $p < 0.01$). The differences were as follows:

Comparison	*p*-value
Dark tube – single tube	< 0.01*
Dark tube – 2 tube	< 0.01*
2 tube – single tube	0.57

*indicates a significant difference

When analysing the difference in the Ct value of the live cells between all treatments, there was a significant difference (χ^2 (4, N=15) = 36.21, $p < 0.01$). The difference were as follows:

Comparison	*p*-value
2 tube (1st tube) – single tube	< 0.01*
Dark tube (1st) – single tube	0.05*
2 tube (2nd) – 2 tube (1st)	< 0.01*
Dark tube (1st) – 2 tube (1st)	< 0.01*
Dark tube (2nd) – 2 tube (1st)	< 0.01*
Dark tube (1st) – 2 tube (2nd)	0.02
Dark tube (2nd) – dark tube (1st)	< 0.01*
2 tube (2nd) – single tube	0.71
Dark tube (2nd) – single tube	0.17
Dark tube (2nd) – 2 tube (2nd)	0.29

*indicates a significant difference

When just looking at the 2nd tube of the two tube method and comparing that to the single tube method, there was no significant difference between using a single tube or two tubes, or using a light sensitive tube (dark) (χ^2 (3, N=9) = 2.29, $p = 0.32$).

9.5.9 Assessment of washing pellet – *Yersinia ruckeri*

Statistical analysis of the difference in the dead cells of the different treatments revealed there was a significant difference (χ^2 (3, N=9) = 12.72, p < 0.01). The differences were as follows:

Comparison	*p*-value
2 x wash – 1 x wash	< 0.01*
No wash – 1 x wash	0.79
No wash - 2 x wash	< 0.01*

*indicates a significant difference

When analysing the difference between the Ct values of the live cells there was no significant difference between the groups (χ^2 (3, N=9) = 1.61, p = 0.45).

When comparing the difference between the live, dead and controls there was a significant difference (χ^2 (4, N=22) = 58.67, p < 0.01). The differences were as follows:

Comparison	*p*-value
Dead_control – Dead	< 0.01*
Live – Dead	< 0.01*
Live_control – Dead	< 0.01*
Live – Dead_control	0.75
Live_control – Dead_control	0.71
Live_control – live	0.71

*indicates a significant difference

9.5.10 Assess optimal parameters in plate format (high-throughput)

Tenacibaculum maritimum

Analysis of the conventional vPCR and qPCR showed no significant differences between the platforms (χ^2 (2, N = 90) = 1.23, p = 0.27), (χ^2 (2, N = 90) = 0.001, p = 0.97) respectively.

ABV

Analysis of the conventional vRT-PCR showed no significant differences between the two platforms (χ^2 (2, N = 72) = 0.72, p = 0.79).

9.5.11 Proficiency testing

Yersinia ruckeri

Two users at the same lab*:* Analysis of the Ct values showed there was no significant difference between the two users (χ^2 (3, N = 80) = 0.002, p = 0.96).

Two users, different lab: The Ct values showed there was no significant difference between the two users (AHL processed immediately, AHL processed day 2, PHEL processed day 2) (χ^2 (3, N = 120) = 2.04, *p*-value 0.36).

Tenacibaculum maritimum

Two users, same lab:

The Ct values from the v-qPCR showed there was no significant difference between the two users (χ^2 (2, N = 76) = 0.14, p = 0.71).

9.6 APPENDIX 6 – INITIAL *MYCOPLASMA BOVIS* VPCR OPTIMISATION

9.6.1 Optimisation

Bacterial strains, media, and growth conditions.

The *Mycoplasma bovis* strain used in this study was from the American type culture collection (ATCC 25523). *Mycoplasma bovis* was grown at 37 °C in Friis broth (FB) for 72 hr. After incubation, bacterial counts were determined by plating cells on Friis agar (FA) after serial 10-fold dilutions. The dilutions 10-5, 10-6 and 10-7 were plated in triplicate and incubated in CO2 at 37°C for seven day prior to colonies being counted and CFU mL-1 determined.

Heat treatment for Mycoplasma bovis.

Dead *M. bovis* cells were prepared by incubating 1 mL of the cell suspension at 100 °C for 10 min. After treatment, the cells were quenched on ice and then brought to RT prior to using. Loss of viability was confirmed by inoculating 100 µL of the dead cell suspension in 1. 9 mL of FB and spread plating 100 µL of the suspension onto FA and incubating at 37 °C without and with CO2 respectively for 7 d.

DNA extraction.

DNA was extracted using the Qiagen DNA mini kit tissue protocol. Briefly, 180 µL lysis (ATL) buffer and 20 µL proteinase K was added to the 200 µL of fluid sample and mixed. This sample was then incubated overnight at 56 °C until lysed. The following day, DNA was extracted as per the manufacturer's tissue protocol.

PCR.

Limit of detection of the assay.

A dilution series was performed and run on the conventional PCR and qPCR in triplicate. The limit of detection (LOD) of the qPCR assay was determined as the level where the best amplification efficiency was achieved (closest to 100 %) and the LOD of the conventional assay was determined as the lowest concentration where all samples amplified. To determine the amplification efficiency, bacterial concentration was plotted against the corresponding cycle threshold (Ct) and the amplification efficiency was assessed using the calculation E = -1+10(-1/slope).

Conventional nested PCR.

A conventional nested PCR previously described by Pinnow et al., (2001) was selected for testing. For the primary and nested round of PCR, DNA was added to 12.5 µL Kapa 2G Fast ReadyMix (2X) (Kapa Biosystems) and 2.5 µM of each primer (primary round: PpMB920-1/ 2, nested round: PpSM5-1/2) to a total volume of 25 µL with nuclease free water. PCR was performed on a Veriti Dx Thermal Cycler (Applied Biosystems). The cycling conditions used were: one cycle of 95 °C for 5 min followed by 40 cycles of 95 °C for 15 sec, annealing at 48°C for 15 sec for the primary round and 55 °C for the nested

round, and 72 °C for one min. DNA extracted from *M. bovis* (ATCC 25523) was used as a positive control and no-template controls of nuclease free water (Sigma, XX) were included in all PCR assays. Resulting amplified material were resolved by electrophoresis in a 0.8 % or 1.5 % agarose gel for the primary and nested round respectively and stained with GelRed (Biotium). The amplicon size produce for the primary and nested PCR were 1911 and 442 bp respectively.

Quantitative PCR (qPCR).

A commercial qPCR kit (VetMAX™ M. bovis Kit, Thermofisher) targeting the polC gene was also performed to quantitate the differentiation between live and dead *M. bovis* cells. The kit was used as per the manufacturer's protocol. Real-time PCR were carried out on the Bio-rad CFX 96 machine (Bio-rad). A positive control provided with the kit and no-template controls of nuclease free water (Sigma) were included in all PCR assays.

PEMAX treatment optimisation.

Dye concentration, incubation temperature of incubation and reaction buffers assessed for optimisation based on the knowledge gained from the aquatic animal pathogens. All optimisation experiments were carried out in triplicate. A stock solution of 2000 µM PEMAX was prepared by adding 500 µL of sterile reverse osmosis water and the solution was kept frozen at -20 °C until used. To determine the optimal dye concentration and incubation temperature, a 200 µL aliquot of dead *M. bovis* at 107, 106 and 105 CFU mL-1 were exposed to final concentrations of 50 and 100 µM of PEMAX dye. These samples were incubated both at room temperature and on ice for 30 min in the dark with agitation of 200 rpm followed by 15 min exposure to PhAST blue light.
The following controls were run in parallel with this experiment:

1) dead *M. bovis* with no treatment at all dilutions,
2) live *M. bovis* with no treatment at 10^7 CFU mL^{-1}
3) live *M. bovis* at 10^7 CFU mL^{-1} exposed to 50 µM dye and not exposed to light,
4) live *M. bovis* at 10^7 CFU mL^{-1} exposed to 100 µM dye and not exposed to light and
5) live *M. bovis* at 10^7 CFU mL^{-1} exposed to light and not exposed to PEMAX dye.

Control 1 was used for a comparison to the treated samples, controls 2 to 5 were used to ensure dye or light had no effect on live cells.

Once the optimal dye concentration and incubation temperature was determined, reaction buffers were trialled to further enhance the differentiation between live and dead *M. bovis* cells. Reaction buffers were trialled on live and dead *M. bovis* at a 10^7 CFU mL^{-1} bacterial concentration only. Differing concentrations of sodium deoxycholate (SD) (0.01 %, 0.001%, 0.0001 %) and reaction buffer + (GenIUL, Barcelona, Spain) (1 X, 0.5 X, 0.25 X, 0.125 X) were used. Controls used in this experiment:

- dead 10^7 CFU mL^{-1} no treatment
- live 10^7 CFU mL^{-1} no treatment

Artificially spiked semen samples. Mixtures of viable and dead *M. bovis* cells were used to assess the suitability of PEMAX treatment with mixed cells in tissue samples. All samples contained the same number of cells only the ratio of viable to dead was adjusted. 100, 80, 60, 40, 20 and 0 % viable cells were mixed with dead cells at two final concentrations of bacteria; 106 and 105 CFU mL-1.
Aliquots (2 x 200 µL) were distributed into vPCR reaction tubes. PEMAX was added to one aliquot and no dye was added to the other. Both aliquots were then processed using the optimised vPCR protocol.

Controls used in this experiment:

- dead 10^7 CFU mL^{-1} no treatment
- live 10^7 CFU mL^{-1} no treatment

Statistical analysis.

Statistical analysis was performed in R studio, version 0.98.501 (R Core Team 2015). A generalised linear model (GLM) was performed. For tests where a qPCR was used, the response variable was the Ct value. These were log transformed to meet the assumption of normality (family = Gaussian). For conventional PCR, the response variable was the binomial result (family = binomial). The specific pair-wise differences between groups was tested using Tukey contrasts and *p*-values were adjusted using the Benjamini & Hochberg method (R package multcomp, Hothorn et al., 2008). The significance of the explanatory variables in all models were assessed using likelihood ratio tests (Zuur et al., 2009). *P-values* < 0.05 were considered statistically significant.

9.6.2 Results

Heat treatment for Mycoplasma bovis.

After heat treatment at 100 °C for 10 min, no growth was detected in any broth suspensions or agar plates of *M. bovis* after incubation for seven days.

Limit of detection.

The LOD of the conventional nested PCR was determined to be 10^1 CFU mL^{-1}. Below this concentration, amplification was not repeatable.
The LOD of the qPCR was also 10^1 CFU mL^{-1} with Ct values of < 35 being unreliable. At this bacterial concentration range (10^1 – 10^7 CFU mL^{-1}), the amplification efficiency was 110 % and increased to 115 % when the range extended to 10 to 10^7 CFU mL^{-1} (Figure 1).

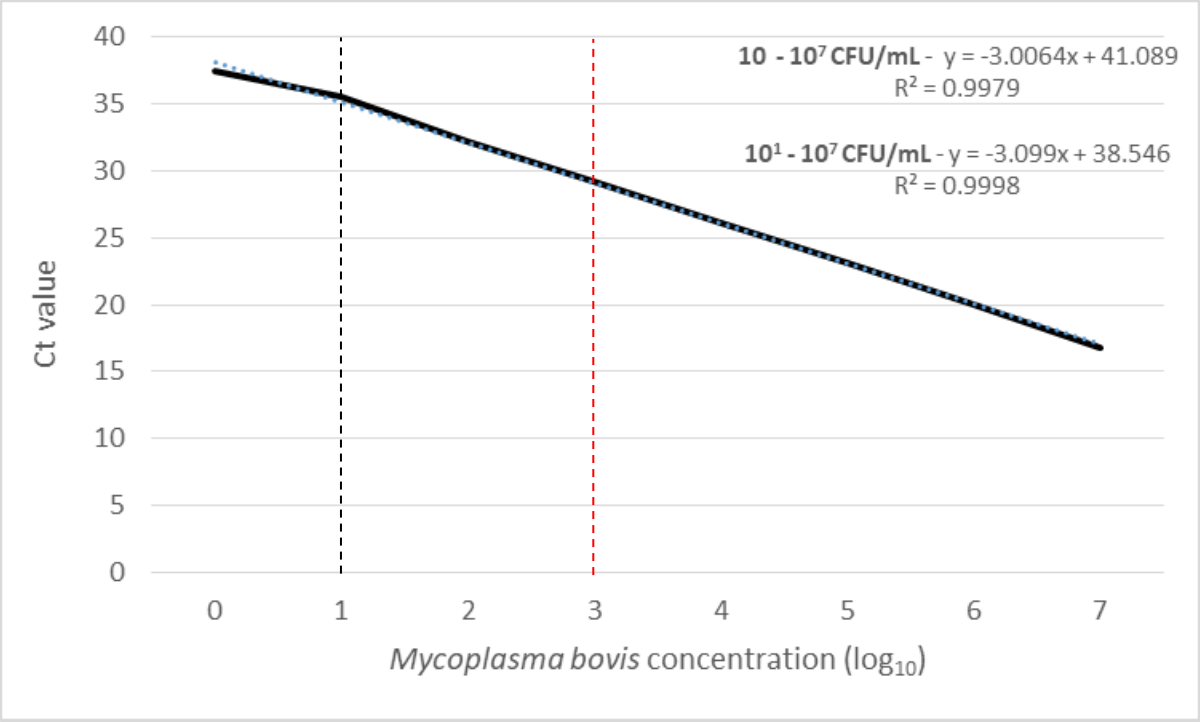

Figure 1. LOD of the M. bovis qPCR and nested conventional PCR (black dashed line) and conventional primary PCR (dashed red line).

PEMAX treatment optimisation.

Optimisation of the dye concentration and incubation temperature revealed a statistically significant difference ($p < 0.05$) between dead *M. bovis* cells treated with PEMAX when compared to cells not treated with PEMAX at both concentrations (Figure 2). No statistically significant difference ($p > 0.05$) was seen between 50 and 100 µM of PEMAX, so the former was chosen for subsequent experiments. Comparing the incubation temperatures, a statistically significant difference ($p<0.05$) was seen when incubating *M. bovis* on ice. No significant difference was seen between any of the other temperatures, thus ice was chosen as the incubation temperature for subsequent experiments. Conventional vPCR results for the different temperature and dye concentrations are shown in Table 1.

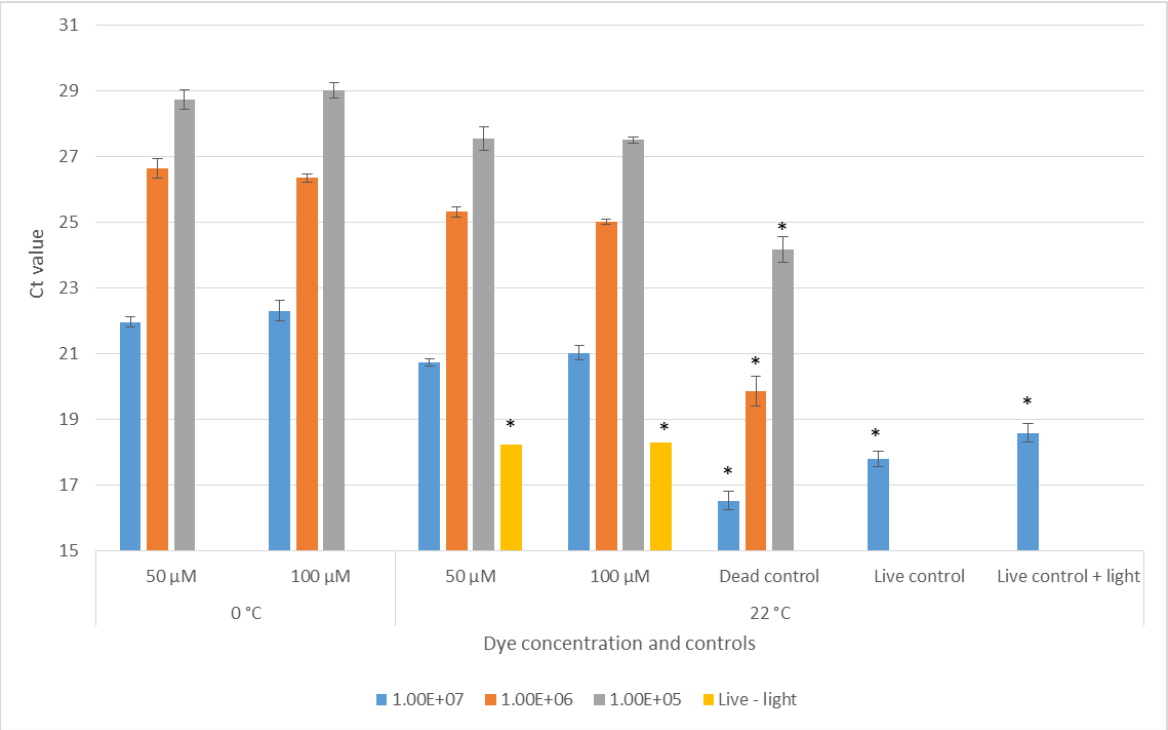

Figure 2. Bars represent average Ct value of dead *M. bovis* exposed to varying concentrations of PEMAX dye with varying incubation times and dye concentrations. * = significant difference from treated samples at both temperatures. Error bars represent the SD between the replicates.

Table 1. Conventional vPCR results for dead *M. bovis* cells at different incubation temperatures two concentrations of dye. Results in the incubation temperature columns indicate the number of replicates out of three that produced an amplicon.

Bacterial concentration (CFU mL^{-1})	**Dye concentration (µM)**	**Incubation temperature (° C)**	
		0	**22**
10^7	50	3	3
10^6	50	1	3
10^5	50	0	3
10^7	100	3	3
10^6	100	1	3
10^5	100	3	3

The use of reaction buffers improved the differentiation between live and dead *M. bovis* cells at all dilutions. Using a 1 X and 0.5 X final concentration of reaction buffer + negatively affected the live cells with high qPCR Ct values compared to the controls and reduced or no amplification of a product by conventional PCR (Table 2. Figure 3). Using reduced concentrations of this buffer reduced the adverse effect on the live cells, however it did not increase the differentiation of live vs dead. The use of SD at a concentration of 0.01 % revealed complete suppression of dead cells in the primary round of the conventional PCR (Table 2) and was shown by real-time PCR to have no effect on live cells (Figure 3).

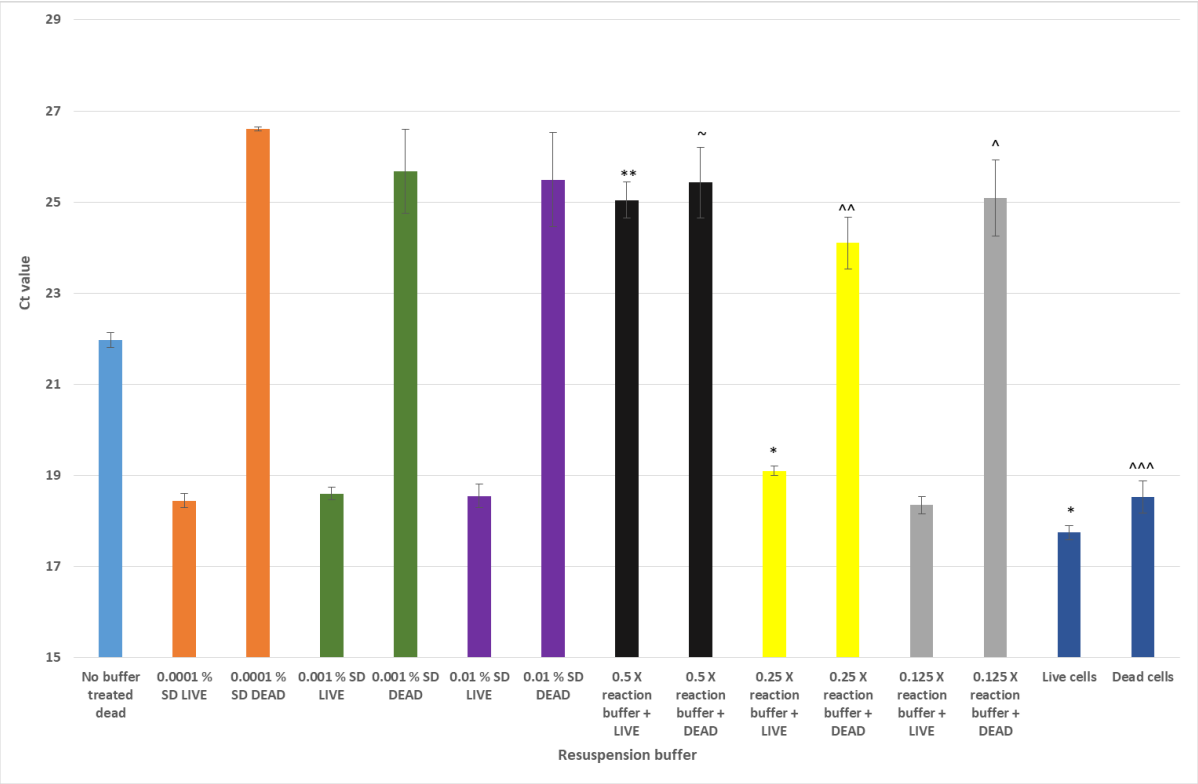

Figure 3. Bars represent average Ct value of live and dead *M. bovis* cells (10^7 CFU mL^{-1}) in different reaction buffers exposed to PEMAX dye. n = 3. * = significant difference from 0.0001 % SD, 0.001 % SD, 0.01 % SD, 0.125 X reaction buffer. ** = significant difference from 0.25 X reaction buffer. ^ = significant difference from 0.0001 % SD. = ^^ = significant difference from 0.001 %, 0.01 % SD. ^^^ = significant difference from 0.125 X reaction buffer, 0.25 X reaction buffer, 0.5 X reaction buffer. ~ = significant difference from 0.25 X reaction buffer. Error bars represent the SD between replicates.

Table 2. Primary and nested results from the conventional PCR results with resuspension buffer at a bacterial concentration of 10^7 CFU mL^{-1}. The number in the 'live' and 'dead' column indicate the number of replicates out of three that showed amplification in the PCR. Red indicates at least one replicate was reduced in intensity on the gel electrophoresis.

Reaction buffer	Live		Dead	
	Primary	Nested	Primary	Nested
0.0001 % SD	3	3	2	3
0.001 % SD	1	3	3	2
0.01 % SD	3	3	0	2
0.5 X Reaction buffer +	3	3	3	3
0.25 X Reaction buffer +	3	3	2	3
0.125 X Reaction buffer +	3	3	3	3

Use of PEMAX optimised protocol to detect dead M. bovis cells in semen.

The protocol optimised using pure cells (50 µM PEMAX, incubation at 0 °C using the reaction buffer 0.01 % SD) was then used on spiked semen samples. Mixed ratios of *M. bovis* that contained live cells revealed detection of DNA in all samples in the qPCR with a trend of reducing Ct value as the amount of live cells in the mix reduced (Figure 4). Samples spiked with dead *M. bovis* cells only revealed reduced amplification in the real-time PCR of an average of 11.9 Ct difference between dead and live cells (=3 log reduction) and showed no amplification in the conventional PCR (Figures 4 and 5).

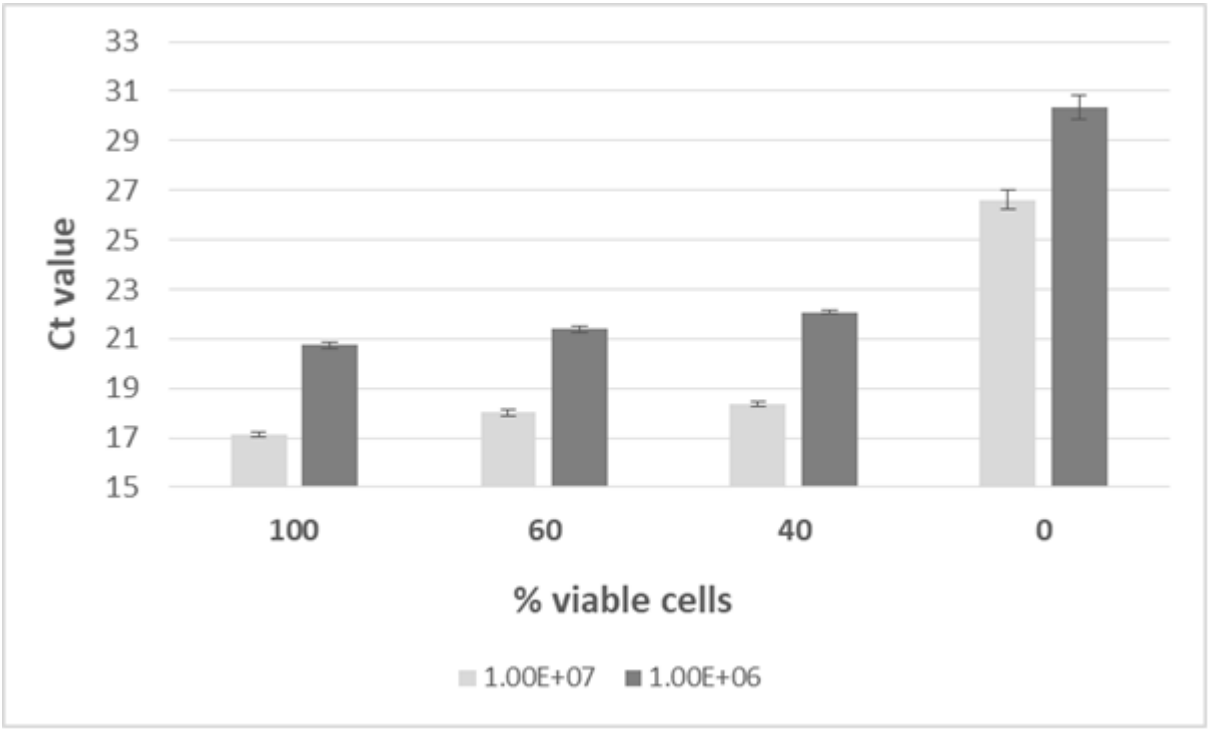

Figure 4. Ct values of samples containing variable amounts of *M. bovis* viable cells in semen matrix. Error bars represent the SD between replicates.

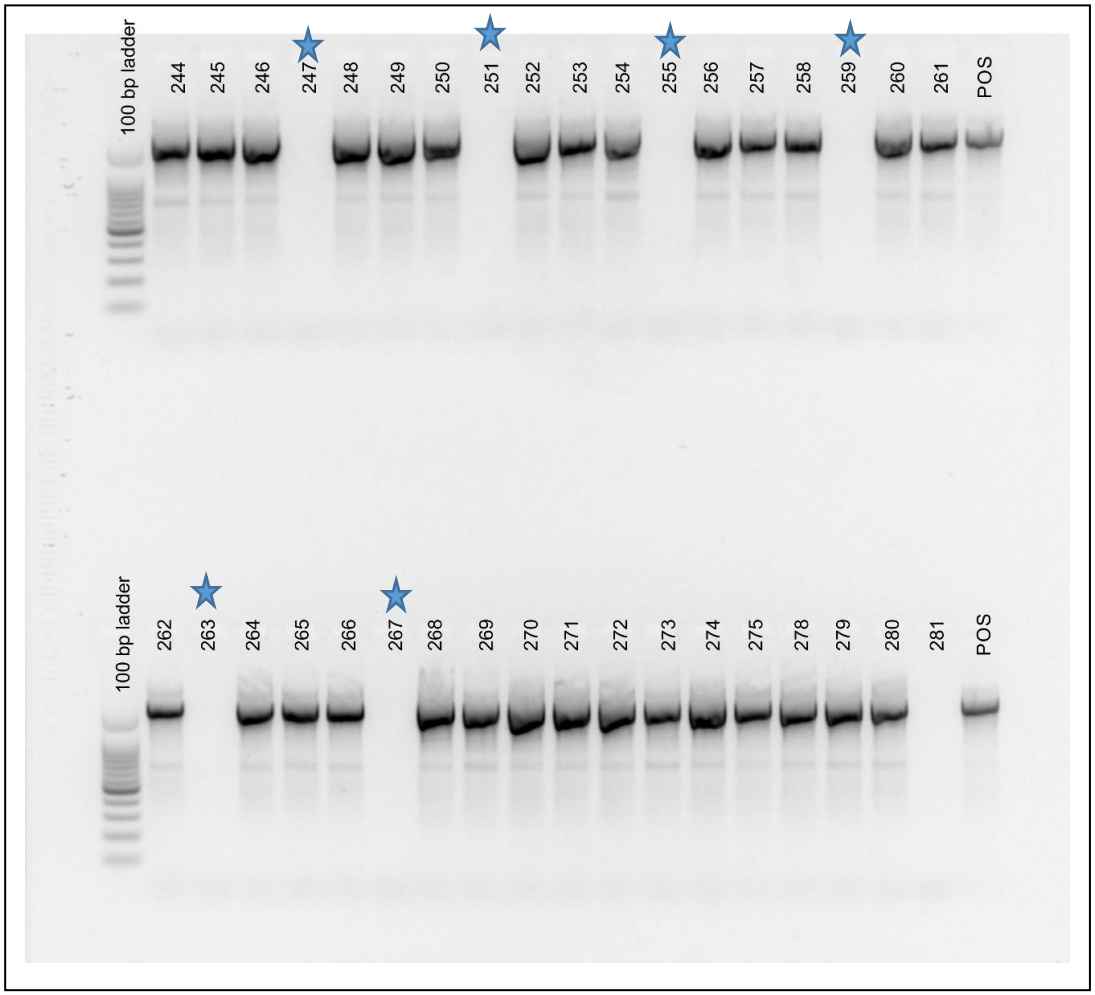

Figure 5. Conventional PCR (1911 bp) gel electrophoresis of samples containing variable amounts of *M. bovis* viable cells in semen matrix. Star = samples that had dead cells only.

9.6.3 Discussion

The aim of the present study was to determine if vPCR could be used on *Mycoplasma bovis*, a bacteria that does not possess a cell wall. The second aim was to determine if vPCR would be appropriate to detect live M. bovis in a matrix of semen.

The initial validation of vPCR using the conventional nested *M. bovis* PCR showed promising results and that full suppression of any amplicon was detected when cell concentrations were above 10^3 CFU mL^{-1}. Full suppression was not seen when using the qPCR. Results from the conventional and qPCR are consistent with previous studies where longer PCR products result in improved efficiency of vPCR (Banihashemi et al., 2012) as well as being consistent with the other pathogens evaluated for vPCR in this project; *Yersinia ruckeri*, *Tenacibaculum maritimum* and ABV. The use of qPCR in conjunction with the cut off value described for *Y. ruckeri* and *T. maritimum* in this project report (Brosnahan et al., 2019a, section 4.17) may be appropriate for *M. bovis*, however further validation work including an increased number of artificially spiked as well as naturally infected tissues would need to evaluated.

Resuspension buffers included in the present study were used to improve efficiency of vPCR. The concentrations of the resuspension buffers used were lower than those used for *Y. ruckeri* and *T. maritimum* (section 4.7). This is likely due to *M bovis* lacking a cell wall and the ability for the surfactants to more readily affect the cell membrane therefore allowing penetration of the dye. This illustrates the importance for consideration of the target pathogen structure when selecting resuspension buffers for evaluation.

Testing of the vPCR on artificially spiked *M. bovis* samples showed the optimised protocol to be effective on concentrations of *M. bovis* 10^5 CFU mL^{-1} and above. To determine the effectiveness on lower concentrations, further optimisation would need to be carried out. Additionally, as the vPCR protocol assessed here had a LOD of 10^3 mL^{-1} detection of concentrations lower than this may benefit from an enrichment protocol as previously described (Brosnahan et al., 2019a).

While vPCR for *M. bovis* was shown to be appropriate in preliminary validation, further testing on a range of bacterial concentrations spiked in semen samples as well as naturally infected samples would need to be evaluated prior to adoption of this technology for these samples.