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IFMA Module 1d - Assessing and predicting emerging *Vibrio* risk

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1 Introduction

Vibrios are Gram negative waterborne bacteria that are considered an emerging pathogen threat in Europe. *Vibrio* spp. are responsible for many important waterborne human diseases, including cholera, primary septicaemia and necrotising wound infections, and gastroenteritis/diarrhoea. Many waterborne bacterial pathogens of human health relevance such as *Vibrio vulnificus* and *Vibrio parahaemolyticus* appear to be proliferating across Europe, and several recent reports have documented increasing *Vibrio* wound infections - in particular linked to heat wave events in the Baltic and North Sea - such as those experienced in 1994, 2003 and 2006 (Baker-Austin *et al*. 2013). During these 'heat wave' years numerous fatalities were also reported in northern European countries linked to these pathogens. The recent change in sea temperature is considered as the most pervasive and severe cause of impact in coastal ecosystems worldwide (Halpern *et al.* 2008), with recent observations demonstrating significant warming in over 70% of the world's coastlines (Lima and Wethey 2012). Several of the fastest warming marine systems anywhere in the world are found in Europe, including the North Sea and Baltic Sea, and it is clear that these areas may be undergoing a transition into 'at risk' regions for disease emergence (Lima and Wethey 2012). Of concern, recent global climate change models (GCMs) suggest sustained and significant warming of coastal regions by the end of the century, including around the UK (Lowe *et al.* 2009). Indeed, a recent publication led by the European Centre for Disease Control (ECDC) placed non-cholera vibrios as the leading group of pathogenic agents sensitive to climate change.

Because pathogenic vibrios appear to grow under select temperature and salinity regimes, this analysis attempted to generate key physio-chemical data to develop, characterise and trial a basic risk-assessment tool for these emerging pathogens. Briefly, a combination of these two variables can be used to build a risk mapping approach, potentially to help identify environmental conditions that drive disease emergence. To perform this analysis accurately, it is necessary to gather relevant data from a variety of sources, including the following parameters:

- Growth optima for given pathogens (temperature and salinity);
- Temperature and salinity data where cases have been reported, as well as from laboratory studies;
- Epidemiological information regarding route of exposure; and
- Detailed climate data prior to, and during reported infections.

This IFMA module aimed to build upon the recently completed Marine Climate Change Centre SEPF project (ME542). This work fits with the NAP action on pathogens (listed on NAP page 143).

2 Methods

2.1 Growth optima experiments

We carried out a comparative analysis of molecular approaches to identify the temperature and salinity optima of *V. vulnificus* – the bacterial species that carries the highest mortality and morbidity burden in reported wound infections in Europe. This was achieved by utilising real-time polymerase chain reaction (PCR) to determine basic growth dynamics associated with these bacteria, using species-specific molecular tests (see Figures 1 a, b). Using this approach it is possible to accurately quantify a large number of bacterial suspensions and so generate a much larger dataset than using growth experiments alone. The PCR-based approach is quicker, easier, has higher throughput, and when analysis time is also taken into consideration, cheaper than traditional methods (e.g. using culture-based, molecular, protein assays, optical density etc.).

For each strain, a set inocula of individual isolates were grown across a range of temperature and salinity gradients (Figure 1 b), then analysed using quantitative real-time PCR utilising a positive control standard for enumeration/quantitation purposes. *V. vulnificus* strains, were grown at different temperature (5-35 ^{o}C) and salinity (0-35 ppt NaCl) for 24 h. A total of 20 *V. vulnificus* strains were chosen for analysis, the majority of which have been implicated with human illnesses to guide clinical relevance, including strains from the USA, Europe and the Far East.

Following incubation, bacterial suspensions were pelleted and boiled prior to analysis. To produce appropriate positive control material for the real-time PCR, the generation of cloned positive PCR was adopted, essentially according to Baker-Austin *et al.* (2011). For real-time PCR experiments, the assay comprised of a total reaction of 25 µl, consisting of 12.5 µl TaqMan Universal PCR Master Mix (Applied Biosystems), 0.45 µl each of forward and reverse primer (as appropriate), *V. vulnificus pilF* primers (100 nM), 5.6 µl nuclease-free water, and 1 µl of probe (500 nM). Five microlitres of template (either plasmids, boiled cell lysate) were subsequently added, and each reaction was performed in triplicate. Amplification was performed using an Applied Biosystems SDS 7900 real-time PCR machine with the following cycling parameters: 1 cycle at 95 °C for 10 min followed by 50 cycles at 95 °C for 15 s and 60 °C for 90 s. For each assay, samples generating a positive reaction result (sigmoid-shaped amplification curve rising above the threshold) in any replicate were considered positive. For each real-time PCR sample replicate, a PCR unit quantity (genome copies of cloned target per reaction) was

calculated using the slope of a standard curve of target DNA, with a C_p value of 42.75 representing the theoretical limit of detection of the assay equalling 1 genome copy of target per PCR reaction. Standard curves for the determination of *V. vulnificus* copy number (per PCR reaction) were constructed using the average of duplicate C_p values encompassing a concentration range of four serially diluted cloned material samples (typically from -4 to -8 dilution of cloned material). Curves with r^2 values of <0.99 were not used for quantification purposes. Analysed TaqMan sample replicates that did generate positive amplification curves were omitted from final analyses, and the average from the three replicates was calculated to give an overall quantity for that sample. To ascertain potential PCR inhibition, a commercially available amplification control was utilised (TaqMan® Exogenous internal positive control reagents, Applied Biosystems), with minor modifications.

Table 1. Real-time primers and probes used in this study

Name	Sequence (5'-3')	Reference
Pil F - F	GATTGACTACGAYCCACACCG	Baker-Austin et al. (2012)
Pil F – R	GRCGCGCTTGGGTGTAG	Baker-Austin et al. (2012)
PilF Probe	$^{(FAM)}$-TGCTCAACCTCGCTAAGTTGGAAATCGATAC-$^{(TAMRA)}$	Baker-Austin et al. (2012)

This quantitative PCR data was then amalgamated to form determine extreme high/low temperature regimes for these pathogens, as well as growth optima (Figure 1b).

2.2 Temperature and salinity data from reported cases

Separately, we carried out an analysis of peer-reviewed publications with an emphasis on published reports regarding temperature and salinity and where possible, reported *Vibrio* infections in north west Europe. These data were reviewed and amalgamated to determine the basic constraints of temperature and salinity for risk assessment purposes. To develop this framework into a full risk mapping approach, it was necessary to obtain accurate sea surface temperature (SST) and sea surface salinity (SSS) data for periods corresponding with previous outbreaks. We utilised datasets from previously published work in this area (Baker-Austin *et al.,* 2013). In particular, we analysed a small number of reported cases alongside long-term SST records (HadISST, Hadley Centre Sea Ice and SST data set, and ERSST, NOAA Extended Reconstructed SST Dataset v3b) as well as shorter-term data from NOAA's Optimum Interpolation v2 Daily SST Analysis dataset, alongside available salinity

datasets. We retrospectively analysed a range of infections during 'at risk' periods using a previously developed web-portal that employs temperature and salinity fields for *Vibrio* risk-assessment purposes (see https://e3geoportal.ecdc.europa.eu/SitePages/VibrioRiskMap.aspx). We also chose to focus on several discrete climatic events that led to increased reporting of *Vibrio* infections. For instance, during 2006, a record number of *Vibrio* wound infections were reported in northwest Europe, and which corresponded with a significant heat wave event (Figure 2).

2.3 Amalgamation of risk runs alongside case data

We carried out a series of 'ground truthing' experiments, whereby we investigated in detail reported instances of *Vibrio* wound infections in NW Europe during anomalous warm weather episodes – such as 1994, 1997, 2003 and 2006; focussing on the relatively few cases where the timing and location of infections was known precisely. The epidemiological data was derived from a combination of peer-reviewed papers, national statistics and grey literature. This data was used to identify key physio-chemical conditions prior to onset of infections and to help inform risk assessment criteria for these pathogens in Europe.

3 Results

3.1 Salinity and temperature analyses

We found a strong correlation with regard to the role of temperature and salinity in determining *V. vulnificus* growth. Both variables interacted significantly, and played an important role in modulating growth in this bacterium (Fig. 1). This growth optima was determined by combining growth data for around 20 strains that were grown in the laboratory across a range of temperature and salinity conditions. This allowed the physio-chemical conditions to be characterised that represent the extremes of growth for this pathogen (e.g. the 'borders'), as well as the growth optima. Individual growth plots for each strain were generated by a quantitative real-time PCR method. There was a small amount of inter and intra strain variability with regard to the growth condition experiments, and this was incorporated into the model. The complexity of this dataset meant that only a simplified visual representation is shown here.

A consistent trend, as shown from the subset of data presented here, indicated that low salinity (< 20 ppt NaCl) and high temperature (> 18 °C) is responsible for higher levels of bacterial proliferation over a 24 hour period. This trend is consistent with data derived from a variety of sources (e.g. peer-reviewed publications) that suggest that these environmental physio-chemical conditions are conducive for growth of this bacterium, and correspond both temporally and spatially with elevated

risk. Highest levels of bacterial growth were observed at 5-15 ppt NaCl salinity and above 20°C. We did find, however, that temperatures above 35°C inhibited growth (data not shown). The molecular

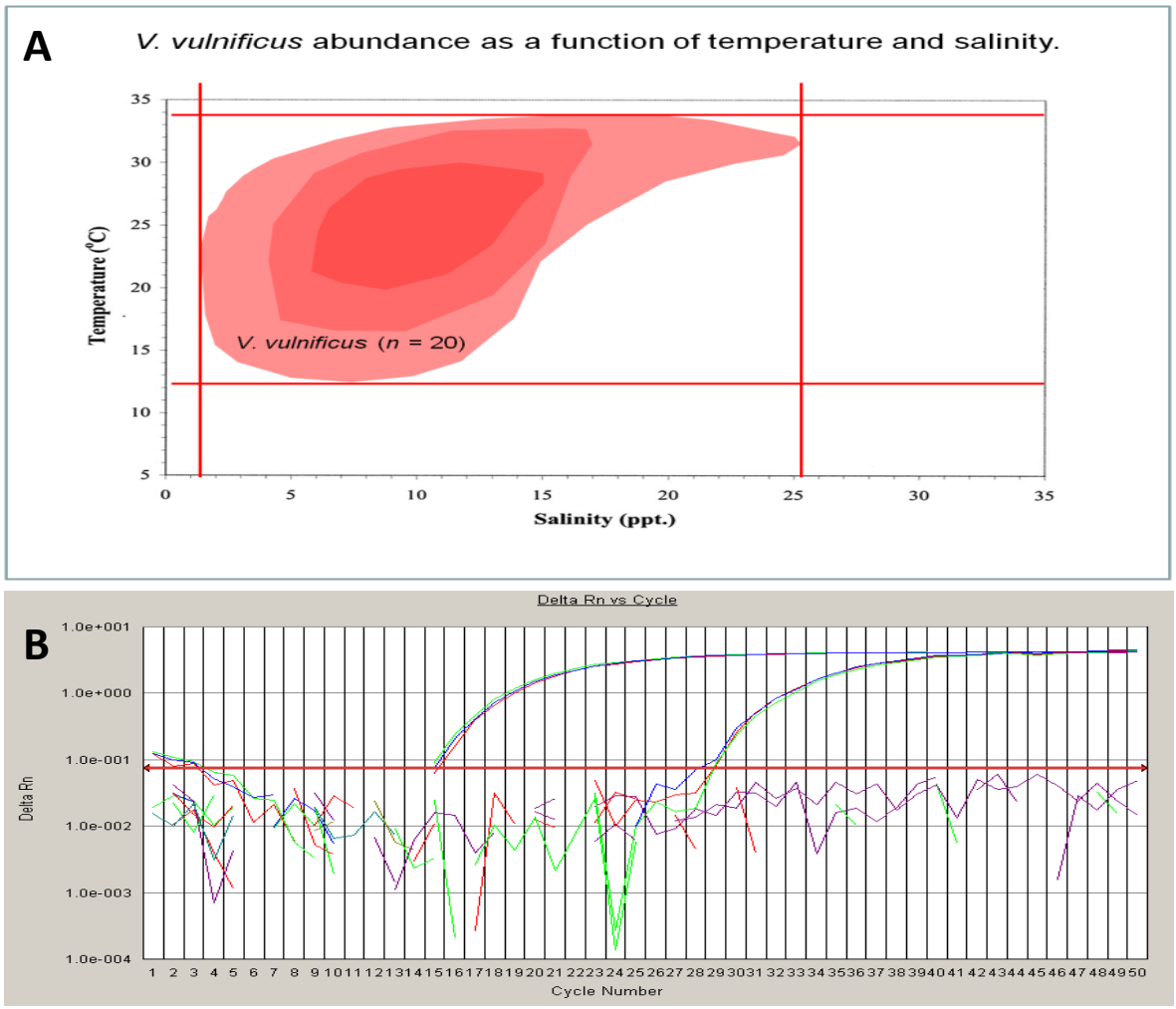

Figure 1. *V. vulnificus* temperature and salinity optima (A), based on the generation of quantitative real-time PCR data (B).

approaches developed to build this quantitative risk-based framework demonstrated consistent reproducibility using replicates of strains, indicating that the method adopted (real-time PCR) was an appropriate and robust methodology in this particular context.

3.2 Temperature and salinity data from reported cases

Using retrospective analysis of epidemiological data as well as SST and SSS we found a clear relationship between anomalous 'at risk' periods and instances of reported disease. The small number of cases that were analysed using this approach should be viewed with some caution (Table 2), but allow us to trial further risk models for downstream analyses. We originally analysed almost 300 reported *Vibrio* infections reported in and around north west Europe from the late 1970s onwards.

We omitted the majority of infections where the specific location and timing of infection/route of exposure was not defined.

Table 2. Example of infection data (subset) used to inform risk mapping approach

Year	Agent	Date and location	Data source
2004	*Vibrio cholerae* (Non O1)	18th-20th July, Sweden (Södertälje)	Ingegard Hokeburg
2006	*Vibrio vulnificus*	7-13th Aug, Sweden (Gotland)	Ingegard Hokeburg
2006	*Vibrio vulnificus*	28-29th Aug Sweden (Dalarö)	Ingegard Hokeburg
2006	*Vibrio cholerae* (non O1)	6th Aug, Sweden (Halland county)	Ingegard Hokeburg
2003	*Vibrio vulnificus*	27-28th Aug, Germany (Luebeck Bay)	Kunt-Lenz (2004)
2003	*Vibrio cholerae* (non O1)	July 20th, Finland (Raahe)	Lukinmaa (2004)
2003	*Vibrio cholerae* (non O1)	Aug 4th Finland (Porvoo)	Lukinmaa (2004)
1994	*Vibrio vulnificus*	Aug 5th Sweden, (South Sweden)	Melhus (1995)

In almost all instances where infections were reported, these corresponded both temporally and spatially with high anomalous SST events during heat wave episodes, and with sustained surface seawater temperatures typically exceeding above 20 °C. We also found a significant relationship with salinity, with cases reported in areas/regions where SSS was below 20 ppt NaCl.

3.3 Amalgamation of risk runs alongside case data

We found a strong relationship between instances of reported infections and 'risk' data captured from our risk mapping approach (see https://e3geoportal.ecdc.europa.eu/SitePages/VibrioRiskMap.aspx for more detail). This allowed us to specifically analyse particular instances of infections reported in north west Europe, and 'ground truth' exposure data with relevant temperature and salinity fields. The model used here uses growth conditions for *V. vulnificus.* This organism was chosen primarily because of its higher pathogenicity potential, and the fact that the majority of recorded fatalities and serious wound infections in Northern Europe are associated with this pathogen.

One of the limitations in our analysis is the paucity of reliable and robust epidemiology data, linking an individual's exposure to a pathogen at a particular time, in a specific location. In a small number of instances, we were able (by using epidemiology data from case reports) to pinpoint a likely transmission route, within a restricted temporal and spatial context, to infer exposure. In these small number of instances, it was striking that the individual was likely exposed at the same time, the vibrio risk mapping approach indicated elevated risk.

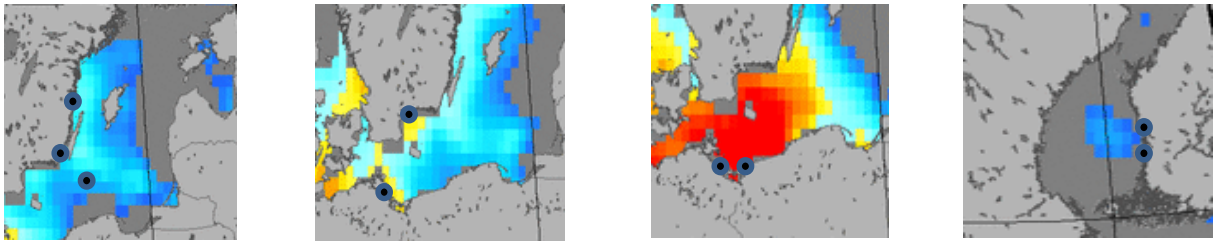

Figure 2. Example screenshots of reported infections (indicated in black) with risk map derived SSS and SST overview fields for a particular day during at risk periods. Fields shown here (from left to right) include western Sweden, southern Sweden and Germany, Germany and Finland.

In general, the data highlighted the utility of risk mapping for generating a rapid assessment during anomalous weather patterns, such as significant heat wave events. The data further confirmed the prior hypothesis that high environmental temperatures and low salinity are likely to be the most significant physio-chemical drivers of risk. The major heat wave years – (2003, 2006 and 1994) account for the majority of infections reported according to our preliminary analyses, and also account for the longest and most sustained risk fields using remote sensing based datasets.

4 Discussion

Emerging infectious diseases are a significant burden on public health institutions, and the transmission of many diseases is determined by many factors, including social, economic and ecological conditions, access to healthcare and intrinsic human immunity (Semenza and Menne, 2009). Central to improving understanding of emerging risk has been the ability to scrutinise previous *Vibrio* outbreaks using retrospective approaches that combine more than one strand of circumstantial evidence - such as those that encompass epidemiological, oceanographic, demographic and microbiological observations. The data reported here merges a range of different approaches, such as information derived from molecular microbiological analyses (Figure 1), epidemiology (Table 2, Figure 2) as well as climatological and risk assessment-based datasets. Effective prevention of waterborne and food-borne pathogens requires detailed epidemiological data to identify likely routes of exposure, risk characteristics (e.g. individuals at particular risk of infections) as well as possible means to disrupt disease transmission. A major challenge regarding the epidemiology of many waterborne pathogens is the role of underreporting in masking the true clinical burden of disease. Indeed, even dedicated surveillance systems set up to monitor specific pathogens record only a small proportion of actual cases (Baker-Austin *et al.,* 2010). The problem of underreporting depends on the

Vibrio species studied, and nationally has significant ramifications for the understanding the basic epidemiology of these pathogens.

Across much of Europe, the risk of *Vibrio* infections is considered to be low. Differences in the quality and coverage of epidemiological datasets across north west Europe must be considered, as too should extrapolation of risk assessments across the entire region. Irrespective, we describe here a potential means of analysing and predicting risk using a free web portal that provides coverage for the entire region. The data underlying this methodology is wholly multi-disciplinary. This approach, which merges the analysis of epidemiology data alongside temperature and salinity datasets is invaluable in identifying areas currently experiencing, and in the future likely to planning waterborne disease risk. The methodology developed and evolved from this work is invaluable in developing near real-time surveillance of shellfish and bathing waters and helping determine appropriate and cost effective options for microbial surveillance in the UK as well as elsewhere. This project also provides an important and detailed evidence base for assessing climate change mediated impacts in coastal regions.

There are several areas where this analysis can be improved and refined further. Owing to the paucity of detailed epidemiology for these pathogens, we chose to use instances where the temporal and spatial data regarding infections and route of exposure was precisely known; this represented a small subset of known cases. To circumvent this limitation, we have also tried to include epidemiologically data from other regions (e.g. the USA, where a more detailed and exhaustive monitoring capacity exists). Secondly, we extrapolated the laboratory experiments (Figure 1) to help refine risk assessment tools, however, more detailed and biologically relevant approaches using field-based experiments would probably represent a more accurate and reliable means of inferring the physio-chemical conditions that drive the abundance of these pathogens in the environment. Finally, we believe that utilising risk modelling approaches coupled to statistical and regional global climate modelling (RGCM) analyses, will be most useful approach to identify regions currently undergoing rapid warming that are likely to experience the greatest risk for these emerging disease by the middle of the 21st century. Given recent RGCM projections for north west Europe, with a concomitant and significant increase in SST and reduction in SSS (Lowe *et al.,* 2009), it is likely that pathogenic vibrios are likely to become a significant threat in this region. The data used to accomplish future risk assessments will likely be a combination of current IPCC and Met Office (Hadley) projection datasets, and should be the focus of future work.

Data are currently being refined using higher resolution models that provide greater granularity for risk mapping purposes. This approach is a first step towards building a generalised risk model for vibrios, and focuses on *V. vulnificus* only. This model will be improved in future incarnations. Indeed, there are some significant differences in the temperature and salinity optima regarding different pathogens. Further development of the models could involve different vibrio species. For instance, *V. vulnificus* grows better in low salinity water, whereas *V. parahaemolyticus* and to a lesser extent, *V. cholera*e, can tolerate higher levels. Several recent studies (e.g. Martinez-Urtaza *et al.* 2012) have identified *V. parahaemolyticus* in open oceanic conditions whereas *V. vulnificus* cannot grow in such environments, and is largely restricted to estuarine and brackish areas. Our intention for future work is the development of separate high resolution risk mapping approaches for *V. vulnificus, V. parahaemolyticus* and *V. cholerae.*

In order for this kind of research to be used in developing real-time risk notices, for example for shellfish mariculture operators, more robust data on potential changes in temperature and salinity, and the possible interactions between these two variables is required. This is an area that Cefas are currently interested in developing alongside our risk models. We have recently collaborated with the Met Office to use regional climate model projection data to identify discrete regions around the coastline of the UK that are likely to warm significantly above 18°C, and with corresponding low salinity (e.g. <30 ppt NaCl).This data will be used to underpin a research paper in this area.

4.1 Consequences of Future Climate Change

Currently in the UK there is very little bivalve shellfish production in areas where these pathogens are currently found. However, under future scenarios suggested by regional climate models, large swathes of the UK coast may warm to an extent that these pathogens become a problem. The inclusion of shellfish harvesting areas into our risk modelling work is a key area that we are currently addressing.

5 Conclusions

The data presented here form the basis of a new set of quantitative risk-based approaches for appraising hazards associated with pathogenic vibrios, in both the UK and north west Europe more widely. In particular, this short project accomplished the following findings:

- Pathogenic vibrios such as *V. vulnificus* grow most effectively at low salinity but high environmental temperatures (< 20 ppt NaCl, > 18 °C). This trend appears to be consistent both within and between strains analysed.
- The experimental data generated from this short project suggests that molecular methods may offer a useful and robust approach for charactering environmental sensitivity or preferences of particular pathogen species.
- Most instances of disease in the past appear to have been linked to heat wave events (e.g. in north west Europe, the summers of 1994, 2003 and 2006). A lack of detailed epidemiology (regionally) precludes us from extrapolating further with regards to risk analysis.
- Remote sensing-based risk mapping approaches, as highlighted here, can be used as a practical means of retrospectively investigating 'at risk' periods and as a possible risk prediction and intervention strategy.

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