Annex to: EFSA's BIOHAZ Panel Scientific opinion "Public health aspects of *Vibrio* spp. related to the consumption of seafood in the EU". doi:10.2903/j.efsa.2024.8896

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Annex B – Food Standards Australia New Zealand information request

B.1. Introduction

Following Australia's largest *V. parahaemolyticus* outbreak in raw oysters[1], an information request was shared with the EFSA Microbiological Risk Assessment (MRA) Network and international partners in 2022. The questions were:

1) Is *Vibrio parahaemolyticus* and/or *V. vulnificus* notifiable human diseases in your country? Have you witnessed an increase in *Vibrio* species illness over the past 3 – 5 years? If so, do you consider that this is climate change related? Is there any substantive correlative evidence for this?

2) What *Vibrio*/environmental monitoring (e.g. sea water temperature, salinity etc.) in shellfish growing areas, if any, do you or your colleagues undertake? Has changes in domestic requirements in your country changed requirements for equivalence testing of imported seafood?

3) What phenotypic methodologies (quantitative and presence/absence) are currently being used for routine identification and testing of vibrios in shellfish? Noting we've witnessed challenges with non-culturable *Vibrio* and issues with most probable number (MPN) methods.

4) Are environmental or human isolates routinely sequenced? Are there specific genes (other than *tdh* and *trh* for *V. parahaemolyticus*) that are of interest from a risk perspective? Are sequences on a public or internal database and what metadata is uploaded associated with those sequences?

B.2. Information received through the MRA network

Replies were received from Croatia, Cyprus, Estonia, France, Germany, Ireland, Norway, Sweden (through MRA representatives).

B.2.1. Question 1: Is *V. parahaemolyticus* and/or *V. vulnificus* notifiable human diseases in your country? Have you witnessed an increase in *Vibrio* species illness over the past 3 – 5 years? If so, do you consider that this is climate change related? Is there any substantive correlative evidence for this?

	Is *Vibrio parahaemolyticus* and/or *V. vulnificus* notifiable human diseases in your country?
Croatia	No
Cyprus	*V. parahaemolyticus* and/or *V. vulnificus* are not notifiable human diseases in Cyprus, only *Vibrio cholerae* is.

[1]https://www.foodstandards.gov.au/industry/FoodIncidents/Pages/Vibrio-parahaemolyticus-and-raw-Pacific-oysters.aspx

Estonia	*V. parahaemolyticus* and/or *V. vulnificus* are not belonging to the notifiable human disease list in Estonia but are registered by Estonian Health Board with specific code under the "Other bacterial or viral intestinal infections" disease category.
France	No. Only the notification of foodborne outbreaks caused by *V. parahaemolyticus* and/or *V. vulnificus* is mandatory.
Germany	In the past, the number of annual human infections associated with *Vibrio* spp. in Germany was reported to be low (~10 cases per year). However, the responsible public health institute (Robert Koch Institute) has been aware of only isolated cases of gastrointestinal infections by non-cholera *Vibrio* since 2000. As human *Vibrio* infections have been notifiable only since March 2020 according to the German Infection Protection Act (Infektionsschutzgesetz (IfSG)), potential cases occurring before 2020 may not have been reliably recognized or recorded and are likely to be higher. It should be noted that only a few investigations of enteropathogenic *Vibrio* were conducted in patients with diarrhoeal diseases in Germany, because of the expected impact of *Vibrio* and the requirement of a highly specific cultivation method for its detection. Currently, comprehensive data are missing to evaluate reliable trends of the *Vibrio* infection situation in humans in Germany and potential effects of the climate change on it. Annual recording of human *Vibrio* infections will help to elucidate the emergence of *Vibrio* in Germany over time. However, further comprehensive monitoring data of aquatic environments and food products derived from them (i.e., fish, seafood, mussels) are necessary for reliable determination of the routes of infection and climate change impacts possibly associated with occurrence of *Vibrio* spp.
Ireland	*V. parahaemolyticus* and/or *V. vulnificus* is not a notifiable disease in Ireland currently. See here for more information on notifiable diseases in Ireland: List of Notifiable Diseases - Health Protection Surveillance Centre (hpsc.ie)
Norway	Clinical *V. parahaemolyticus* and *V. vulnificus* are not routinely sequenced. All *Vibrio* species are mandatorily notifiable in MSIS since 2019, however the Norwegian Institute of Public Health (NPHI) has reference function only for *V. cholerae* and *V. parahaemolyticus*. Therefore, clinical laboratories in Norway are not required to send other *Vibrio* species isolates to NPHI. NPHI have sequenced all *Vibrio* isolates received from 2014-2018 as part of a research projects involving more country in the Nordic and Baltic area. The study has been published in Eurosurveillance: https://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2022.27.28.2101088. The sequences of this study are available in a public database as referred in the publication.
Sweden	Yes, they are both notifiable since 2004.

	Have you witnessed an increase in *Vibrio* species illness over the past 3 – 5 years?
Croatia	Not applicable
Cyprus	There wasn't any increase in *Vibrio* species illness the last 3 – 5 years.
Estonia	According to our knowledge there is no increase of *Vibrio* caused infections in Estonia.
France	Yes, the number of non-cholera *Vibrio* infections is increasing in France (an average of 10 cases per year between 1995-2016, 25 cases in 2017, 59 in 2018, 67 in 2019, 47 in 2020, 46 in 2021). *V. parahaemolyticus* is since 2018 the most frequently reported species, mainly associated with moderate forms of gastroenteritis following seafood consumption. *V. cholerae* (non-O1/non-O139 strains) is the second most frequently reported species, but with a high level of imported cases contracted in countries with poor hygiene and sanitation standards. To be noted the emergence of *V. fluvialis* since the last five years, associated with gastroenteritis.
Germany	
Ireland	Not aware of clinical surveillance or relevant research projects in this area. *Vibrio* species are not - currently - a problem around the coast as our water temps are too low.
Norway	
Sweden	There is a slight tendency to increased numbers over the last ten-year period; however, this might as well be due to an increased awareness amongst physicians. The

	maximum number of reported cases (178) was in 2018, which was a warm summer (by Swedish standards). The majority of cases are swimming related, mainly ear infections in kids but also some wound and blood infections has been recorded (Vibrioinfektioner – sjukdomsstatistik — Folkhälsomyndigheten (folkhalsomyndigheten.se)). Find more information on Nordic cases between 2014-2018 in this paper: eurosurv-27-28-2.pdf (eurosurveillance.org)

	If so, do you consider that this is climate change related? Is there any substantive correlative evidence for this?
Croatia	Not applicable
Cyprus	Not applicable
Estonia	
France	The significant increase in the number of non-cholera *Vibrio* infections could be explained by the high temperatures reported in France since 2017, especially during the summer season (REPHY 2017-2020 data, Santé publique France analyses), but also by the evolution of diagnostic methods in laboratories, Multiplex PCR and Mass Spectrometry, which have improved surveillance thanks in particular to better detection of less symptomatic cases. Both aspects are certainly involved.
Germany	
Ireland	
Norway	
Sweden	

B.2.2. Question 2: What Vibrio/environmental monitoring (e.g. sea water temperature, salinity etc.) in shellfish growing areas, if any, do you or your colleagues undertake? Has changes in domestic requirements in your country changed requirements for equivalence testing of imported seafood?

	What *Vibrio*/environmental monitoring (e.g. sea water temperature, salinity etc.) in shellfish growing areas, if any, do you or your colleagues undertake?
Croatia	Nothing obligatory at the national level. It may be that some scientific research is done in Adriatic Sea, from time to time. These results have no influence on possible monitoring measures, or other requirements on national level.
Cyprus	There is no shellfish farming in Cyprus, therefore no such monitoring.
Estonia	
France	There is no routine monitoring in shellfish production areas, but occasional prevalence studies have been carried out. At the European level, there are no microbiological criteria defined for *Vibrio* in foods. The requirements are therefore national (microbiological limit: absence in 25 g). Analyses may be carried out in certain contexts (but not routinely), particularly on imports. The management rules in case of detection are the same for imported and domestic products.
Germany	Currently, comprehensive monitoring on the occurrence, diversity, and impact of *Vibrio* bacteria in ecosystems used for the production of fish, seafood or other aquatic-derived food products in Germany does not exist. Basic research studies, conducted in the past and ongoing, indicate low risks for the occurrence of human pathogenic *Vibrio* species carrying the respective hemolysin genes (*V. parahaemolyticus*) or toxins (*V. cholerae*) as primary virulence factors originating from food products and water. However, further studies on geographic differences, periodic changes and climate effects need to be conducted. A yet unpublished study on *V. parahaemolyticus* occurrence from imported shrimps conducted by our own laboratory highlights the introduction of toxinogenic and multidrug-resistant (Extended Spectrum Beta-Lactamase (ESBL)- and

	carbapenemase-producing) isolates to Germany via seafood imports and requires further attention and management actions (i.e. broader monitoring purposes). To be prepared for a possible increase of *Vibrio* bacteria occurrence and its impact on human health based on changing climate conditions (climate change) in Germany and worldwide, monitoring of *Vibrio* species abundance needs to be considerably extended to a "One Health" approach in the future.
Ireland	There is no routine monitoring programme for *V. parahaemolyticus* in classified production areas for bivalve shellfish in Ireland. The Marine Institute collects environmental data for sea water temperature, salinity, etc. from coastal areas however the sampling locations may not be in all classified production areas and is not collected with *V. parahaemolyticus* in mind.
Norway	On the environmental side, there are some research studies carried out by the Institute of Marine Research in Norway. One published last year: https://pubmed.ncbi.nlm.nih.gov/32558371/. There is also another project focusing on *V. parahaemolyticus* in mussels which was part of a Norwegian University of Life Sciences MBU master thesis which Senior Advisor Ettore Amato from our department at NPHI was external sensor for (not sure the thesis is already public available).
Sweden	Temperature and salinity are measured in approximately half of the shellfish growing areas, not for *Vibrio* management specifically.

	Has changes in domestic requirements in your country changed requirements for equivalence testing of imported seafood?
Croatia	
Cyprus	
Estonia	
France	
Germany	
Ireland	We are not aware of such requirements. We don't do any routine monitoring, nor do we routinely check imported seafood, as we wouldn't import too many oysters.
Norway	
Sweden	

B.2.3. Question 3: What phenotypic methodologies (quantitative and presence/absence) are currently being used for routine identification and testing of vibrios in shellfish?

	What phenotypic methodologies (quantitative and presence/absence) are currently being used for routine identification and testing of vibrios in shellfish? Noting we've witnessed challenges with *non-culturable Vibrio and issues with MPN methods?*
Croatia	Not applicable
Cyprus	There is no routine testing for *Vibrio* in food, but the Veterinary Services would test seafood samples on an ad hoc basis e.g in case of a Rapid Alert System for Food and Feed (RASFF) notification.
Estonia	Standard method EVS EN ISO 21872-1 is used in Estonia, but very few food analyses are performed in Estonia per year, and all of these have been only for exported food items.
France	The detection of *Vibrio* in shellfish is done according to the standardized method NF EN ISO 21872-1 (search for the 3 species *V. parahaemolyticus, V. cholerae and V. vulnificus*). The strains are confirmed either by biochemical or molecular biology techniques. At the national reference laboratory, confirmations are made by PCR with a search for the pathogenicity genes *tdh* and *trh* for *V. parahaemolyticus* and CTXA and CTXB for *V. cholerae.*

Germany

The current accepted phenotypic methods for the detection of the three vibrio species with the highest human impact (further mentioned as human pathogenic vibrios) *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* are provided by both the Food and Drug Administration (FDA) and the International Standards Organisation (ISO).

The ISO method ISO 21872-1:2017 (Microbiology of the food chain - Horizontal method for the determination of *Vibrio* spp. - Part 1: Detection of potentially enteropathogenic *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus*) describes a horizontal method for detection of the mentioned species from products intended for human consumption and for use in animal feed. Environmental samples, taken in the context of food production or handling of food products, can also be analyzed. The standard is subdivided into four key steps: primary and secondary enrichment in a liquid selective medium, isolation and identification, and confirmation. The isolation of human pathogenic *Vibrio* spp. from food products can be improved by the application of various incubation temperatures, depending on the target species as well as the state of the food matrix to be investigated. As examples, the recovery rate for species *V. parahaemolyticus* and *V. cholerae* in fresh products is enhanced by enrichment at 41.5°C, while *V. vulnificus* as well as *V. parahaemolyticus* and *V. cholerae* are more successfully enriched at 37°C in deep-frozen, salted or dried products. Samples often contain only a small number of *Vibrio* bacteria and are frequently accompanied by a large number of other bacteria. Accordingly, a two-stage selective enrichment process is carried out for their growth in alkaline saline peptone water (ASPW). The halo- and alkali-tolerant properties of vibrios are exploited in order to suppress accompanying flora. Two solid selective culture media are then inoculated from the two enrichment stages in order to isolate and confirm individual colonies. To suppress Gram-positive/-negative bacteria, a thiosulfate-citrate-bile salts-sucrose (TCBS) agar is used, which simultaneously permits a differentiation between sucrose utilizing (*V. cholerae*, *V. metschnikovii*, *V. fluvialis*, *V. furnissii* and *V. alginolyticus*) and other species (*V. mimicus*, *V. parahaemolyticus* and *V. vulnificus*). A solid culture medium complementary to TCBS is used as the second solid medium. CHROMagar™ *Vibrio* (CVA) is frequently utilized here, which contains a proprietary mixture of colorants. This serves as a substrate for the β-galactosidases from *V. parahaemolyticus* (color: mauve), which is colored to contrast strongly with *V. cholerae* and *V. vulnificus* (turquoise) and other *Vibrio* species (colorless to creamy, *V. alginolyticus*). This enables the species *V. parahaemolyticus* to be identified/distinguished, since it exhibits the same morphology on TCBS as *V. mimicus* and *V. vulnificus*. Other media can be applied for the identification of other species. The use of cellobiose polymyxin B colistin (CPC) agar, for example, permits the identification of *V. vulnificus*.

Finally, presumptive vibrio bacteria need to be confirmed by suitable biochemical and/or molecular methods (esp. PCR). However, matrix assisted laser desorption ionization – time of flight (MALDI TOF) mass spectrometry can also be used for *Vibrio* identification/typing but has not been accounted by ISO 21872 as a potential method for species confirmation yet. Further determination of virulence-affecting determinants is currently primary conducted by specific PCRs (especially for *V. parahaemolyticus* and *V. cholerae*), but whole-genome sequencing (WGS)-based purposes provide a deeper insight into the complexity of pathogenicity factors (i.e., *V. vulnificus*).

As mentioned above, the second phenotypic method is provided by the FDA in the FDA's Bacteriological Analytical Manual (BAM, chapter 9). The scope of application is restricted to foods and cosmetics. While the detection of *V. cholerae* and *V. vulnificus* is largely comparable with the ISO standard, two additional detection options are described for *V. parahaemolyticus*. By utilizing a hydrophobic grid membrane filter (HGMF), the sample can be concentrated in the first method and then applied to various solid selective culture media. The second method is a plating method, which makes use of DNA probes to identify the overall *V. parahaemolyticus* population as well as pathogenic, *tdh*-positive colonies. A modified version of the latter method is also described in another ISO standard (ISO/TS 21872-2:2020: Microbiology of the food chain – Horizontal method for the determination of *Vibrio* spp. – Part 2: Enumeration of total and potentially enteropathogenic *Vibrio parahaemolyticus* in seafood using nucleic acid hybridisation). Neither FDA's nor ISO/TS 21872-2:2020, Part 2) are routinely used, because of its expenditure of time.

Ireland	In Europe the predominant method used to enumerate *V. parahaemolyticus* is ISO 21872:2017 which involves an initial selective enrichment in alkaline salt peptone followed by direct plating on TCBS agar. Presumptive colonies are isolated before being subjected to molecular tests to determine species identification and the presence of putative pathogenicity markers (*tdh* and *trh* genes).
Norway	
Sweden	Sweden does not have any routine surveillance or control of *Vibrio* in shellfish (yet).

B.2.4. Question 4: Are environmental or human isolates routinely sequenced? Are there specific genes (other than *tdh* and *trh* for *V. parahaemolyticus*) that are of interest from a risk perspective? Are sequences on a public or internal database and what metadata is uploaded associated with those sequences?

	Are environmental or human isolates routinely sequenced?
Croatia	Not applicable
Cyprus	Environmental or human isolates are not sequenced
Estonia	Routine sequencing for *Vibrio* isolates is not performed in Estonia
France	The national reference laboratory has been systematically sequencing strains of clinical origin for several years. Only sequences published in scientific articles are made public. They systematically search for the *hemolysin* genes*, tdh* and *trh,* and recently for the genes of the secretion factors T3SS 1 and 2, but this remains very preliminary and any significant association with the presence of these latter genes has been made. The only criteria taken into account from a risk perspective remain the *tdh* and *trh* genes. There is no surveillance and therefore no sequencing of environmental strains.
Germany	Based on the expected increase of the impact of human pathogenic *Vibrio* species in the future, our laboratory has decided to sequence (paired-end short read Illumina sequencing) all *V. cholerae* isolates carrying *ctx* (Cholera toxin gene), *V. parahaemolyticus* carrying *tdh* or *trh* (*V. parahaemolyticus* hemolysin genes) as well as all *V. vulnificus* isolates (due to its multifactorial virulence factor components) provided from our collaborating federal state laboratories. Furthermore, we also subject other *Vibrio* spp. isolates to WGS, especially if they were expected to be associated with human infections, undesired diagnostic phenotypes or phenotypic properties. In general, WGS data are assessed for their quality and analysed for basic genus-/species-specific features (i.e., multilocus sequence types (MLST), average nucleotide identity (ANI), antimicrobial resistances (AMR) etc.). Regarding pathogenicity evaluation, we use determinants included in the VFDB database for *Vibrio* spp. (http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Vibrio) comprising a complex set of genes involved in adherence (chitin-regulated pilus: *V. vulnificus;* Fimbrial low-molecular-weight protein pili: *V. vulnificus*; N-Acetylglucosamine (GlcNAc)-binding protein A; immunogenic lipoprotein A: *V. vulnificus*; multivalent adhesion molecule 7: *V. parahaemolyticus*; mannose-sensitive hemagglutinin pili; OmpU: *V. vulnificus*; toxin-coregulated pilus; *V. parahaemolyticus* adhesive factor: *V. parahaemolyticus*), effector delivery systems (extracellular protein secretion; T3SS1; T3SS2; VAS T6SS), motility components (Flagella), exotoxins (accessory cholera enterotoxin; cholix toxin; cholera toxin; multifunctional autoprocessing RTX toxin; thermostable direct hemolysin: *V. parahaemolyticus*; thermolabile hemolysin: *V. parahaemolyticus;* TDH-related hemolysin: *V. parahaemolyticus*; *V. cholerae* cytolysin; *V. vulnificus* hemolysin: *V. vulnificus*; Zonula occludens toxin), exoenzyme (Hemagglutinin protease; *V. cholerae* neuraminidase), biofilm components (autoinducer-2; biofilm-associated Protein 1; cholerae autoinducer-1; rugosity and biofilm modulator A and C; *Vibrio* polysaccharide) and other factors (Accessory colonization factor). Up to now, WGS are primary stored on our in-house BfR server. Respective datasets we release to public repositories upon publication of case reports, research studies and genome announcements. However, we are also supporting the exchange of WGS data

	to our collaborators upon request. Up to now, the released WGS include only the minimal set of metadata necessary for uploading to SRA or Genbank (i.e. sampling year, source, country, etc.). If available, further metadata can be provided upon individual request of researchers to our laboratory. Disclaimer: The consultant laboratory for *Vibrio* is part of the Unit Diagnostics, Pathogen Characterisation and Food borne Parasites (HoU: Dr. Martin Richter, martin.richter@bfr.bund.de). The head of the laboratory is Dr. Jens-André Hammerl (Jens-Andre.Hammerl@bfr.bund.de).
Ireland	Not applicable
Norway	
Sweden	No, isolates are not routinely sequenced, but some information is included in the supplementary material in the paper above.

	Are there specific genes (other than *tdh* and *trh* for *V. parahaemolyticus*) that are of interest from a risk perspective?
Croatia	Not applicable
Cyprus	
Estonia	
France	
Germany	
Ireland	
Norway	
Sweden	

	Are sequences on a public or internal database and what metadata is uploaded associated with those sequences?
Croatia	Not applicable
Cyprus	
Estonia	
France	
Germany	
Ireland	
Norway	
Sweden	

B.3. Info received through international collaboration

Replies were received from U.S. Food and Drug Administration (US FDA), Canadian Food Inspection Agency (Canada), New Zealand NZ Ministry for Primary Industries (MPI, New Zealand), Food Safety Commission of Japan (Japan), and Food Standards Australia New Zealand (FSANZ; Australia).

B.3.1. Question 1: Is *V. parahaemolyticus* and/or *V. vulnificus* notifiable human diseases in your country? Have you witnessed an increase in *Vibrio* species illness over the past 3 – 5 years? If so, do you consider that this is climate change related? Is there any substantive correlative evidence for this?

	Is *V. parahaemolyticus* and/or *V. vulnificus* notifiable human diseases in your country?
US FDA	Yes. Vibriosis has been nationally notifiable since 2007 through the Cholera and Other *Vibrio* Illness Surveillance (COVIS) system.
Canada	*See attached report
New Zealand	Not notifiable disease, only cases where there is a suspected common source are notifiable. *Vibrio* has been included in the foodborne disease annual report since 2020 only.
Japan	The incidents of *V. parahaemolyticus* food poisoning in Japan are collected by the Ministry of Health, Labour and Welfare (MHLW) of Japan. Mandatory notification complying with the Food Sanitation Law. ("The statics of food poisoning"). On the other hand, the incidents of *V. vulnificus* food poisoning in Japan are not collected specifically. The annual number of *V. parahaemolyticus* food poisoning incidents having shown a decreasing tendency in recent years in Japan. To reduce the number of *V. parahaemolyticus* food poisoning incidents, the MHLW established specifications and standards for seafood safety from the production stage to the consumption stage in June 2001. In addition, low temperature storage of seafood and suitable food handling were recommended by the MHLW, especially for seafood-related businesses and consumers. After that, the number of *V. parahaemolyticus* food poisoning incidents was successfully reduced.
Australia	*V. parahaemolyticus* is notifiable in the Northern Territory, Tasmania, South Australia and Western Australia but not in Queensland, New South Wales, Australian Capital Territory or Victoria. *V. vulnificus* is notifiable in the Northern Territory and Tasmania only.

	Have you witnessed an increase in *Vibrio* species illness over the past 3 – 5 years?
US FDA	In 2020 (the most recent year for which data are available), the incidence of vibriosis had decreased 25% from the 2017-2019 average. Previous years' data is difficult to compare as starting in 2017, reporting of cases included Culture Independent Diagnostic Testing (CIDT) results as well as culture confirmation.
Canada	
New Zealand	Yes, including outbreaks
Japan	
Australia	It is hard to determine whether there has been an increase in illness or an increase in reporting. Illnesses were reported prior to 1992 (Hall, 1993), but not consistently after that. Outbreaks have noted since 2016 (Harlock et al.; 2022), with one significant outbreak associated with raw oysters from South Australia in 2021 which triggered a national incident response: Vibrio parahaemolyticus and raw Pacific oysters from Coffin Bay, SA (foodstandards.gov.au)

	If so, do you consider that this is climate change related? Is there any substantive correlative evidence for this?
US FDA	N/A
Canada	
New Zealand	There is certainly a link, although we haven't strong evidence (data) for that.
Japan	
Australia	A link is suspected, but no scientific evidence as yet. The 2016 outbreak in Tasmania was associated with a marine heat wave, and the 2021 outbreak in South Australia was associated with unseasonably warm water in early spring.

B.3.2. Question 2: What *Vibrio*/environmental monitoring (e.g. sea water temperature, salinity etc.) in shellfish growing areas, if any, do you or your colleagues undertake? Has changes in domestic requirements in your country changed requirements for equivalence testing of imported seafood?

	What *Vibrio*/environmental monitoring (e.g. sea water temperature, salinity etc) in shellfish growing areas, if any, do you or your colleagues undertake?
US FDA	From a regulatory perspective, *Vibrio* and environmental monitoring are not required in shellfish growing areas. However, there are some state regulatory agencies that do routinely monitor specific *Vibrio* levels (either *V. parahaemolyticus* or *V. vulnificus*) in shellfish. In general, water and air temperature, as well as salinity are documented along with the *Vibrio* levels in shellfish. In addition, numerous researchers (primarily academic) monitor *Vibrio* levels in water and shellfish and in recent years have been including more environmental data with their studies (nutrient levels, chlorophyl a, turbidity, etc.).
Canada	
New Zealand	No ongoing monitoring, however, two environmental surveys have been performed in 2020 and 2021, where environmental data have been collected (water temperature, salinity, rainfall in 2020 and same plus turbidity, pH, phytoplankton, chlorophyll a in 2021).
Japan	
Australia	Those areas in Australia that have had issues with *Vibrio* spp. monitor water temperature as a guide to presence. Several short term (<3 year) surveys have been conducted, but not in all growing areas or all states.

	Has changes in domestic requirements in your country changed requirements for equivalence testing of imported seafood?
US FDA	The short answer here is "no". Specifically for molluscan shellfish, we continue to require an MOU or equivalency agreement for trade of raw bivalve molluscs.
Canada	
New Zealand	No changes at the moment, there are no domestic testing requirements
Japan	
Australia	No changes at the moment. A significant body of work is being undertaken on this currently and this will help inform any future requirements for testing of imported (and domestic) seafood.

B.3.3. Question 3: What phenotypic methodologies (quantitative and presence/absence) are currently being used for routine identification and testing of vibrios in shellfish?

	What phenotypic methodologies (quantitative and presence/absence) are currently being used for routine identification and testing of vibrios in shellfish? Noting we've witnessed challenges with *non-culturable Vibrio and issues with MPN methods?*
US FDA	There are multiple types of methods available in the FDA BAM and the National Shellfish Sanitation Program (NSSP). Additionally, a wider variety of methods is used by academic researchers. Current regulatory methods are specifically targeted to total *V. vulnificus*, as well as total and pathogenic *V. parahaemolyticus*. As with any testing approach, they each have their advantages and limitations. 1. The direct plating method involves spreading dilutions of shellfish homogenate onto agar plates. The growth is transferred to a filter by colony lift and the filter is hybridized with specific probes.

	2. MPN-culture methods are used, where selective agar plates are streaked from turbid MPN tubes and typical colonies selected for confirmation. Isolate confirmation can be done by biochemical identification (reliability of this approach is not the best) or molecular testing (above mentioned colony hybridization, PCR, etc.). 3. MPN-real-time PCR methods are used where crude DNA preps are made from aliquots of turbid MPN tubes. Presence/absence of specific targets is determined by real-time PCR.
Canada	
New Zealand	PCR – MPN method used for the surveys
Japan	
Australia	Routine testing for vibrios in Australia has typically been conducted in accordance with either the Australian Standard (AS 5013.18) which describes a horizontal cultural method for presence/absence and an MPN m for enumeration, the US FDA Bacteriological Analytical Manual or the presence/absence method described in ISO/TS 21872-1. The AS 5013.18 has recently been withdrawn due to lack of available validation data. Concerns have been raised about the availability of an enumerative method with validation status that is accessible for laboratories. There are also significant concerns for the detection of VBNC cells with culture-based methods due to the extremely low presence of vibrios in implicated samples.

B.3.4. Question 4: Are environmental or human isolates routinely sequenced? Are there specific genes (other than *tdh* and *trh* for *V. parahaemolyticus*) that are of interest from a risk perspective? Are sequences on a public or internal database and what metadata is uploaded associated with those sequences?

	Are environmental or human isolates routinely sequenced?
US FDA	The GenomeTrakr and PulseNet networks include sequencing of both human and environmental *V. parahaemolyticus* isolates. However, there is limited participated from these network labs in sequencing of *V. parahaemolyticus*. In addition, some researchers (including those at FDA, CDC, and other federal agencies) sequence new and historical isolates from their collections.
Canada	
New Zealand	Almost all the environmental and human isolates obtained during outbreaks for the last 3 years have been sequenced, and we will continue
Japan	
Australia	Environmental isolates are not routinely sequenced, this would be on a case-by-case basis and dependent on the investigator. Human isolates may be sequenced but this information is not readily available.

	Are there specific genes (other than *tdh* and *trh* for *V. parahaemolyticus*) that are of interest from a risk perspective?
US FDA	This is still an area of much research interest. Some genes have been identified for specific strains/sequence types (STs) that are targeted regionally. However, the NSSP still relies on *tdh* and *trh* for regulatory decisions.
Canada	
New Zealand	Maybe but research is still ongoing, current methods used are still based on *tdh* and *trh*
Japan	
Australia	

	Are sequences on a public or internal database and what metadata is uploaded associated with those sequences?
US FDA	All sequences generated through FDA and GenomeTrakr are available on the NCBI Pathogens Database. At a minimum, metadata includes isolate source (clinical or environmental), year of isolation, and location (country). Many isolates have additional metadata associated with them.
Canada	
New Zealand	Internal database
Japan	
Australia	It is possible sequences are on an internal database – likely AusTrakka (https://www.auspathogen.org.au/, https://portal.austrakka.net.au/)

Canadian Food Inspection Agency
Agence canadienne d'inspection des aliments

Information request – FSANZ
Vibrio parahaemolyticus
September 2022

Canada

Request received from FSANZ – Re: Vibrio
Response prepared by the Canadian Food Inspection Agency (CFIA), Public Health Agency of Canada (PHAC), Health Canada, Bureau of Microbial Hazards (HC-BMH)

1) Is *Vibrio parahaemolyticus* and/or *V. vulnificus* notifiable human diseases in your country? Have you witnessed an increase in *Vibrio* species illness over the past 3 – 5 years? If so, do you consider that this is climate change related? Is there any substantive correlative evidence for this?

In Canada, *Vibrio parahaemolyticus* (Vp) and *V. vulnificus* are not considered nationally notifiable diseases. *V. parahaemolyticus* is found naturally in Canadian coastal waters, with higher concentrations detected in the summer (June-September). Over the past several years, the Public Health Agency of Canada has observed a pattern in Vp case load, with the annual number of clinical cases peeking during the summer months when there is a higher abundance of V. *parahaemolyticus* within marine costal environs due to sea surface temperatures being at their highest during this period.

The number of locally acquired cases tends to decrease as seawater temperatures drop during the fall and winter months. Since these diseases are not nationally notifiable however, it is difficult to assess if there have been changes in Vp case counts over time.

There is considerable scientific evidence in the existing body of literature to suggest that climate change and warming ocean temperatures are affecting the clinical burden of these pathogens:

- Baker-Austin, C., Stockley, L., Rangdale, R. and Martinez-Urtaza, J. (2010), Environmental occurrence and clinical impact of *Vibrio vulnificus* and *Vibrio parahaemolyticus*: a European perspective. Environmental Microbiology Reports, 2: 7-18. https://doi.org/10.1111/j.1758-2229.2009.00096.x
- Baker-Austin, Craig, et al. "Emerging Vibrio risk at high latitudes in response to ocean warming." *Nature Climate Change* 3.1 (2013): 73-77.
- Martinez-Urtaza, J., Bowers, J. C., Trinanes, J., & DePaola, A. (2010). Climate anomalies and the increasing risk of Vibrio parahaemolyticus and Vibrio vulnificus illnesses. *Food Research International*, *43*(7), 1780-1790.

Warming ocean temperatures and other climate anomalies brought on by climate change may significantly extend the seasonal period and geographical range of *Vibrio parahaemolyticus* and/or *V. vulnificus* thus contributing to an increase in disease incidence in Canada, especially during the warmer months.

2) What Vibrio/environmental monitoring (eg sea water temperature, salinity etc) in shellfish growing areas, if any, do you or your colleagues undertake? Has changes in domestic requirements in your country changed requirements for equivalence testing of imported seafood ?

Question 2a: What Vibrio/environmental monitoring (e.g. sea water temperature, salinity etc.) in shellfish growing areas, if any, do you or your colleagues undertake?

At the federal level, the Public Health Agency of Canada has undertaken seasonal environmental monitoring of seawater temperature using real-time data provided by the National Oceanic and Atmospheric Administration. Beginning in the late spring (Approximately June 1st), seawater temperature is tracked on a weekly basis for several pre-determined locations near or adjacent to shellfish harvesting areas. Once the average daily seawater temperature reaches a set temperature threshold of 15°C, it is expected that the number of locally acquired Vp cases will begin to rise, as Vibrios become more abundant within coastal waters, fish and shellfish.

Seawater temperature monitoring is also carried out in the fall (starting in late September and continuing into October) to determine when water temperatures have fallen below the temperature threshold and locally acquired case activity may begin to decrease as a result. In both scenarios, the Public Health Agency of Canada works with various other federal and provincial partners to explore potential risk mitigation measures, and to help increase the public's awareness of Vibrio in shellfish during the time of year when the risk is highest.

In Canada, Safe Food for Canadians Regulations-licensed Operators are required to develop, document and implement preventive controls. For ready-to-eat bivalve molluscan shellfish such as live oysters, this includes controls to prevent contamination with, and growth of, *Vibrio parahaemolyticus* (Vp). In short, validated controls must be in place to ensure that bacteriological guidelines can be met in the end product.

Given that environmental factors influence the initial load of Vp in shellfish being harvested, processor controls typically include measuring water/shellfish meat temperatures, and sometimes salinity at the harvest site. An indication of general presence/level of Vp during risk periods is often assessed with monitoring samples. Often, additional validated control measures (e.g. purging) are employed to ensure that the initial load of Vp in harvested product is low enough that the final product is able to meet guidelines.

In Canada, requirements for Vp control are outlined in "measures to control the risk of *Vibrio parahaemolyticus* in live oysters" and "validation of preventive controls for *Vibrio parahaemolyticus*".

The Canadian Food Inspection Agency performs regular inspections to verify that preventive control measures are in place, are validated, and are effective. The CFIA also has surveillance sampling plans for *Vibrio parahaemolyticus* in oysters (shellstock only), destined for raw consumption, at registered domestic shellfish establishments and importers. Currently, there are approximately 200 samples planned each year. Results are published annually at https://inspection.canada.ca/food-safety-for-industry/food-chemistry-and-microbiology/food-safety-testing-bulletin-and-reports/eng/1453324778043/1453327843364.

The Vibrio research lab of HC-BMH conducts testing of *Vibrio* spp in seafood as part of various research projects. Since 2015, over 100 bivalve mollusc samples representing Atlantic Canada harvest sites, and also representing products sold at retail were tested for an ongoing project. This lab also tested over 200 samples of bivalve molluscs sampled at retail level as part of FoodNet Canada surveillance in 2018-2019. Finally, one of the projects of this lab has been to test over 100 imported warm water shrimp samples from April 2015 to March 2022 to see AMR burden, and for monitoring clinically significant *Vibrio* spp.. In general, the HC-BMH research lab keeps track of water temperature at the time of sampling, if possible, as well as salinity, to associate the outcome of their lab analyses

Question 2b: Have changes in domestic requirements in your country changed requirements for equivalence testing of imported seafood?

Since 2015, the CFIA has enforced a Vp guideline of:

Guideline for *Vibrio parahaemolyticus* in raw oyster shellstock (end product) intended for raw consumption						
Test organism	**Product type**	**Number of sample units (n)**	**Acceptance number (n)**	**m (MPN/g)**	**M**	**Criteria for action**
Vibrio parahaemolyticus	Raw oyster shellstock	5	0	100	n/a	Reject if any unit is equal to or exceeds m (i.e., ≥ 100 MPN/g)

The *Safe Food For Canadians Regulations* came into force January 15, 2019, and are generally consistent with the previous requirements for preventive controls in the former *Fish Inspection Regulations* (i.e. negligible change to domestic requirements).

Required import controls for oysters include:

1) The live or raw (frozen or unfrozen) molluscan shellfish were harvested in an authorized country of harvest and are a species approved to be exported to Canada and;
2) The live or raw (frozen or unfrozen) molluscan shellfish were handled and processed by an authorized shipper / establishment.

For more information, please refer to: Importing live and raw molluscan shellfish - Canadian Food Inspection Agency (canada.ca)

Generally, a SFC-license and Preventive Controls are required to import bivalve molluscan shellfish. Importers are required to implement controls to ensure that the above-noted guidelines are met in imported product. More information can be found at Importer Licence requirements, as well as preventive control requirements. The import process is described here: import process.

3) What **phenotypic** methodologies (quantitative and presence/absence) are currently being used for routine identification and testing of Vibrios in shellfish? Noting we've witnessed challenges with non-culturable Vibrio and issues with MPN methods.

The CFIA uses a combination of phenotypic and genotypic approaches for analyzing shellfish for Vibrio. As the Canadian standard is quantitative as shown above, agency labs currently analyze samples using an MPN method based on USFDA BAM Chapter 9: Vibrio.
Colony confirmation is conducted using the qPCR method MFLP-102 Identification of *Vibrio parahaemolyticus* colonies by real-time polymerase chain reaction, which assays for the TLH species marker as well as the TRH and TDH pathogenicity markers.

In the private sector, a review of accredited scopes indicate the use of MFLP-37 (MPN), USFDA BAM Chapter 9 (MPN, Membrane filtration), and AOAC PTM 050902 (BAX qPCR for *V. cholerae*, Vp, and *V. vulnificus*).

The MFLP methods can be found in Health Canada's Compendium of Analytical Methods Volume 3. They are attached and also available upon request at https://www.canada.ca/en/health-canada/services/food-nutrition/research-programs-analytical-methods/analytical-methods/compendium-methods/laboratory-procedures-microbiological-analysis-foods-compendium-analytical-methods.html

Non-culturable Vibrio is an issue of which the CFIA is aware. CFIA researchers are working on this issue and potential avenues to improve our analytical methods, with some research potentially to be published later this year or next.

We would appreciate any research or data you would also be willing to share regarding non-culturable Vibrio or specific MPN method issues.

The Health Canada study has been using a phenotypic method using 'direct plating after resuscitation' (J.AOAC Int. (2017)), which can quantify the viable *Vibrio* spp responsible for causing illnesses. This method is not able to enumerate very low levels of bacteria, for which MPN method is suitable.

4) Are environmental or human isolates routinely sequenced? Are there specific genes (other than *tdh* and *trh* for Vp) that are of interest from a risk perspective? Are sequences on a public or internal database and what metadata is uploaded associated with those sequences?

The CFIA does not regularly sequence Vibrio isolates obtained from diagnostic testing for food samples.

That being said, the CFIA has initiated a research project in partnership with the British Columbia Centre for Disease Control (BCCDC) to sequence and characterize a large volume of historical and contemporary Vibrio isolates obtained from shellfish samples and other sources. The work is currently under way and we would anticipate one or more publications detailing any results of this project once complete. Details of these collaborative projects can be found here:

https://www.genomebc.ca/blog/new-research-will-broaden-canadas-ability-to-identify-strains-of-oyster-related-illnesses

https://www.genomebc.ca/projects/unified-pathogen-control-one-health-approach-specifically-targeting-vibrio-upcoast-v

As for public vs internal database, all CFIA sequence data is maintained in our internal databases with all associated metadata (location of sampling, all sample details such as date of sampling, isolation date, etc).

Typically when CFIA uploads sequence data to a public platform (such as NCBI) it is with limited associated metadata. Further metadata may be available upon request for publically available sequences, depending on the nature of the request. At this time it has not yet been determined whether the WGS data obtained from the Vibrio sequencing project will be uploaded to a public facing platform.

For clinical samples (human isolates), PHAC routinely sequences any *Vibrio parahaemolyticus* and/or *V. vulnificus* received at the National Microbiology (NML) laboratory, but not all isolates are currently forwarded to NML for WGS by the provincial and territory labs. We do upload representative isolates to NCBI, primarily to facilitate comparison with CDC, when uploading to NCBI the metadata included is as follows:

WGS ID	Organism	Collected By	Collection Date	Geo_loc_name	Host_disease	Host	Isolation Source	Lat_lon
PNCVXXXXXXX	Vibrio parahaemolytiaus	PHAC	YYYY-MM-DD	Canada	Missing	Homo sapiens	Stool, blood, etc.	Missing

If we detect a cluster of *V. parahaemolyticus*, then we do run in-silico prediction for tdh and trh, we do not look for other genes routinely. Both *V. parahaemolyticus* and *V. vulnificus* analysis is SNV based; we do not have a validated wgMLST scheme to use

From a research perspective, a Canadian study published in 2014 (looking at clinical data from 2000-2009) found that although t*dh* and *trh* were predominantly detected in clinical strains of *V. parahaemolyticus* (Vp), there were reports of clinical Vp testing negative for *tdh* and *trh*, indicating involvement of other toxins. From our Canadian clinical Vp cases we had found 4% of the isolates to be

without *tdh* and *trh* (J.Clin.Microbiol.(2014), suggesting risk factors may not be confined to *tdh* and *trh* gene expression. This field is very active, particularly investigating the contribution of the two secretion systems, T3SS and T6SS. Also, Vp specific ToxR may be a factor.