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## **Bacterial foodborne *Vibrio* infections: health risk assessment of the occurrence of *Vibrio* spp. (non-cholera vibrios) in food**

BfR Opinion No 011/2022 of 13 April 2022

Primarily found in bodies of salt or brackish water as well as wetlands, *Vibrio* bacteria are widely prevalent in the environment worldwide. These bacteria are frequently responsible for the bacterial contamination of seafood, including fish and fish products. If these foods are eaten raw or are not heated adequately before consumption, the vibrios they contain can give rise to diarrhoea in humans. *Vibrio* propagation is also favoured by the global rise in ocean temperatures. The German Federal Institute for Risk Assessment (BfR) is of the opinion that the risk of foodborne infections from consumption of seafood contaminated with pathogenic vibrios will increase eventually.

Mussels and oysters, which are sedentary organisms that feed by filtering seawater in their environment, may contain higher concentrations of *Vibrio* bacteria. Most of the commercial products available have either been heated or treated using other methods (e.g. marinating, smoking, drying or salting) and should therefore contain a low concentration of bacteria. For people with a weakened immune system or pre-existing medical conditions such as chronic liver diseases, consumption of live oysters can present a health risk through foodborne infections caused by vibrios. This is particularly true if these foods are contaminated with toxigenic (*trh/tdh*-positive) *Vibrio parahaemolyticus* isolates or *Vibrio vulnificus*.

A requirement to communicate human infections with vibrios to the Robert Koch Institute (RKI), was introduced in Germany in April 2020. This means that there are no official German statistics available on cases of foodborne illness caused by the consumption of seafood containing vibrios up to this time. Therefore, no reliable statements about their incidence rate are currently possible. Given the minimal level of exposure, however, the health risk is considered to be low. This assessment, however, may change in the future depending on changing climatic conditions in the next years as well as improvements in data collection.

The BfR advises consumers to ensure that all seafood dishes are adequately heated during preparation. Vibrios are safely killed off if food is heated to interior temperatures of at least 70 °C for two minutes. Ensuring that general hygiene rules are followed when storing and preparing food ('good kitchen hygiene') can also contribute effectively to protection against foodborne infections.

BfR risk profile: Health risk assessment of the occurrence of <i>Vibrio</i> spp. in food (Opinion number [number/year])				
<b>A</b> Affected persons [1]	General population		People with chronic conditions	
<b>B</b> Probability of an impairment to health from the consumption of contaminated seafood that has not been adequately heated	Very low	Low	<b>Medium[1]</b>	High Very high
<b>C</b> Severity of impairment to health from the consumption of contaminated seafood that has not been adequately heated	No impairment	Mild impairment [reversible/irreversible]	<b>Moderate impairment [reversible/irreversible] [2]</b>	Severe impairment [reversible/irreversible]
<b>D</b> Validity of available data	High: The most important data are available and are internally consistent		<b>Medium: Some important data are missing or contradictory [3]</b>	Low: A large volume of important data is missing or inconsistent
<b>E</b> Controllability by the consumer	Controls not needed	<b>Controllable with precautionary measures [4]</b>	Controllable through avoidance	Not controllable

Fields with a dark blue background indicate the properties of the risk assessed in this opinion (for more details, see the text of Opinion number [number/year] from the BfR dated [day/month/2021]).

**Explanations**

The risk profile is intended to visualise the risk outlined in the BfR Opinion. The profile is not intended to be used to compare risks. The risk profile should only be read in conjunction with the corresponding Opinion.

**[1] Row B – Probability of an impairment to health**

No data are available on the quantitative contamination of seafood in Germany by the three enteropathogenic *Vibrio* species that most commonly cause gastrointestinal infections in humans (*Vibrio parahaemolyticus*, *Vibrio cholerae non-O1, non-O139* and *Vibrio vulnificus*). The level of exposure to *Vibrio* bacteria in food products for German consumers is therefore unknown.

**[2] Row C – Severity of impairment to health**

Infections are self-resolving and of average severity. Symptoms include diarrhoea with abdominal cramps, nausea, vomiting, headaches and mild fever, and typically last for three days on average in patients with a healthy immune system (Nair et al., 2007).

**[3] Row D – Validity of available data**

No data are available on the quantitative contamination of seafood in Germany by the three enteropathogenic *vibrio* species. The level of exposure to *vibrios* in food products for German consumers is therefore unknown.

**[4] Row E – Controllability by the consumer**

A health risk to consumers from foodborne infection results primarily from raw and insufficiently heated food products. As a general rule, the pathogens are safely killed off if food is heated to at least 70 °C and this interior temperature is maintained for at least two minutes.

**1 Subject of the assessment**

The German Federal Institute for Risk Assessment (BfR) has prepared a health risk assessment of the occurrence of *Vibrio* spp. (non-cholera vibrios) in food in Germany. Typically found in bodies of salt or brackish water as well as wetlands, *Vibrio* bacteria are widely prevalent in the environment worldwide. As a result, they are often the cause of bacterial contamination of seafood, including fish and fish products, which can cause diarrhoea and similar symptoms following consumption. As global ocean temperatures continue to rise, these kinds of infections are likely to increase in Germany as well. In this context, following questions were addressed:

1. Which species within the bacterial *Vibrio* genus (non-cholera vibrios) are, within the context of foodborne illnesses, of paramount importance as human disease causative

- agents for the investigation of food potentially contaminated with pathogenic vibrios (non-cholera vibrios)?
2. Which information is available to the BfR on the prevalence and importance of pathogenic vibrios (non-cholera vibrios) in seafood products sold in the retail sector, and in mussels and oysters produced in Germany?
  3. Which trends can be determined from figures on the prevalence of foodborne illnesses as caused by the occurrence of pathogenic vibrios (non-cholera vibrios) in food?
  4. What are the parameters that promote the occurrence, propagation and transmission of pathogenic vibrios (non-cholera vibrios) in food?
  5. Which strategies are considered efficient in terms of minimising the occurrence of pathogenic vibrios (non-cholera vibrios) in food, which relate to the cold chain, and the preservation and decontamination methods utilised during the production process (including processing, transportation and storage), and which can influence the contamination of food with pathogenic vibrios (non-cholera vibrios)?
  6. Which standardised culture techniques and molecular protocols are suitable for the detection and analysis of the health risk posed by pathogenic *Vibrio* spp. (non-cholera vibrios) in food?
  7. Which markers/indicators (e.g. genes or proteins) are suitable – e.g. as virulence factors of pathogenic strains – for facilitating (rapid) detection of pathogenic vibrios within food production? With what level of confidence can these markers indicate a health risk associated with the consumption of food contaminated with vibrios (non-cholera vibrios)?

The present health risk assessment has focused on the bacteria referred to as ‘non-cholera vibrios’, since only these particular types have been detected as bacterial contaminants in seafood in Germany. The answers to the questions raised are presented in section 3.3 of this health risk assessment.

## 2 Results

A hazard to consumer health from food results primarily from raw and insufficiently heated food products. Mussels and oysters, which are sedentary organisms that feed by filtering the seawater in their environment, may contain higher concentrations of bacteria. In bodies of salt water, vibrios are to be expected as a normal member of the natural bacterial flora. Accordingly, these bacteria are present in fish and (in higher concentrations) in bivalve molluscs. Environmental conditions play an important role. As a general observation, rising water temperatures and decreasing salt content may be associated with an increase in the level of contamination with vibrios. For people with a weakened immune system or pre-existing medical conditions such as chronic liver diseases, consumption of live mussels/oysters can present a serious risk of infection caused by vibrios, particularly if the food in question is contaminated with the species *Vibrio vulnificus*.

Seafood is consumed by all population groups, including the higher-risk population groups as described above. Most products available have either been heated or treated using other methods (marinating, smoking, drying, salting, etc.) and should therefore contain lower levels of bacteria. As a general rule, the pathogens are safely killed off if food is heated to at least 70 °C and if this interior temperature is maintained for at least two minutes. No cases of vibriosis following the consumption of seafood have been reported in Germany to date. A notification requirement for cases of vibriosis was not introduced in Germany until 2020. The re-

sponsible public health institute, the Robert Koch Institute, notes on its website that the Institute has been aware of only isolated cases of gastrointestinal infections by non-cholera vibrios since 2000.

Details:

1) No data are currently available on the quantitative contamination of seafood in Germany by the three enteropathogenic *Vibrio* species that most commonly cause gastrointestinal infections in humans (*Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus*). The level of exposure to *Vibrio* bacteria in food products for German consumers is therefore unknown.

2) It can be assumed that adequate heating (interior temperature of 70 °C maintained for two minutes in the food) results in the inactivation of all pathogenic vibrios. Any uncertainty about the risk from excessive vibrio contamination is therefore mainly related to those products that are consumed raw or after brief cooking only. Such foods include oysters in particular and several other types of mussels, as well as fish consumed raw, as is the case with sushi or sashimi. Only sporadic cases of gastrointestinal infections resulting from contaminated oysters and other mussels have been reported in Germany. Additionally, the quantities consumed per capita are comparatively low. Therefore, it may be assumed that, even for these types of foods, currently exposure to pathogenic vibrio bacteria is so low that the probability of occurrence of impairments to health after consumption of these products can also be considered as low.

3) The uncertainty arising from a lack of reliable data on the quantitative contamination of oysters and mussels by vibrios is considered low, in view of the low rates of reported infections. However, it should be remembered that vibrio infections have only been a notifiable disease in Germany since 2020. This lack of mandatory notification also means that very few investigations of enteropathogenic vibrios were conducted in patients with diarrhoeal diseases in Germany. Potential cases of vibrio infections could therefore not or only very rarely be recorded, especially since these bacteria require highly specific culture methods for detection. It is possible that true cases of gastrointestinal vibrio infection were not recorded.

4) A quantitative characterisation of enteropathogenic vibrios in oysters/mussels would be one approach suitable for determining uncertainty in relation to health risks. For characterisation of this type, culture protocols (MPN method) or real-time PCR methods could be applied in order to detect enteropathogenic vibrios.

### 3 Rationale

#### 3.1 Risk assessment

##### 3.1.1 Hazard identification

###### 3.1.1.1 Vibrios as natural inhabitants of aquatic environments

The Vibrionaceae family comprises gram-negative, non-spore-forming bacteria that occur naturally in aquatic ecosystems such as oceans, estuaries and aquaculture throughout the world. The Vibrionaceae family consists of several genera, of which the *Vibrio* (*V.*) genus contains the most important pathogenic species for humans. This genus has more than 100 member species, of which roughly a dozen species have the potential to cause disease in humans. These pathogens can cause both intestinal and extra-intestinal infections (such as wound and ear infections) (Baker-Austin et al., 2018; Ceccarelli et al., 2019).

As climate change and the associated rise in ocean temperatures are expected to increase the occurrence of *Vibrio* bacteria in their natural habitats and a rise in *Vibrio* infections are therefore also predicted (Baker-Austin et al., 2012; Baker-Austin et al., 2017).

As vibrio concentrations in aquatic ecosystems increase infections resulting from direct contact with contaminated water will play a significant role. These infections occur in warmer seasons in particular as a result of leisure activities such as bathing in or hiking next to expanses of coastal water. As a result, extra-intestinal infections (such as wound and ear infections) caused by human pathogenic *Vibrio* species are regularly reported in Germany during warmer parts of the year (RKI, 2020). Extra-intestinal infections are not considered further in this risk assessment, since they are not typically associated with the consumption of food products. A few isolated cases of wound infections suffered by employees working in food production as a result of handling raw seafood products have been reported in the literature (Bisharat et al., 2005).

However, this risk assessment focuses primarily on gastrointestinal *Vibrio* infections resulting from the consumption of fish, fishery products and seafood, including molluscs and crustaceans. Food of marine origin is referred to collectively by the term 'seafood' in the following. Infections can result when these kinds of food are not adequately heated before consumption or are consumed raw.

The *Vibrio* species that most frequently cause seafood-borne gastrointestinal infections in humans are *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus*. According to estimates from expert committees at the World Health Organisation (WHO) and the Food and Agriculture Organisation of the United Nations (FAO), these three pathogens are the most significant in terms of risks to public health. As a result, risk assessments have already been published for these three species by the FAO and WHO in relation to global trade with seafood products (FAO/WHO, 2010a; 2020).

In the course of this risk assessment, two further *Vibrio* species will be considered, which have often been detected in seafood samples in Germany by official food analysis laboratories and sent to the Consultant Laboratory for Vibrios at the BfR for confirmatory analysis. In addition to the three *Vibrio* species mentioned above, *V. alginolyticus* and *V. metschnikovii* will therefore also be considered.

Table 1 lists the *Vibrio* isolates from food as received by the Consultant Laboratory for Vibrios during the period 2017 to 2020.

**Table 1:** *Vibrio* isolates from food received by the Consultant Laboratory for Vibrios during the period 2017 to 2020, with *Vibrio* species as detected

Matrix	Total	<i>V. para-haemolyticus</i>	<i>V. cholerae</i>	<i>V. vulnificus</i>	<i>V. alginolyticus</i> *	<i>V. metschnikovii</i>	<i>V. fluvialis</i>	<i>V. furnissii</i>	<i>V. mimicus</i>	<i>V. Harveyi</i>	<i>V. campbellii</i>	Other <i>Vibrio</i>	Non- <i>Vibrio</i>	Origin (country)
Oysters	54	48 (10 trh)	–	–	4	–	1	–	–	–	–	–	1	DE (North Sea)
Common mussels	402	303 (5 trh)	24	1	45	10	1	2	–	–	–	12	4	North Sea DE, NL, North Atlantic
Other mussels	16	12	2	–	2	–	–	–	–	–	–	–	–	DE (North Sea) (12)
Brown shrimp	125	33	–	1	70	5	1	–	–	4	1	5	5	DE (North Sea)
Prawns, common shrimp, etc.	244	133 (6 trh)	26	3	24	11	1	–	1	6	12	13	14	BD (45), EC (61), HU (15), IN (32), TH (21), VN (38)
Other shellfish	14	9	2	–	–	2	–	–	–	–	–	1	–	BD (1), EC (2), FR (1), IN (2), VN (2)
Fish, fish products	43	4	5	1	–	27	2	1	2	–	–	–	1	VN (9), NO (1), DE (2), IN (3), GR (1), Indian Ocean (1)
Squid, squid products, etc.	2	1	–	–	1	–	–	–	–	–	–	–	–	No data
Others	2	–	1	–	–	–	–	–	–	–	–	1	–	EC (1)
<b>Grand total</b>	<b>902</b>	<b>543</b>	<b>60</b>	<b>6</b>	<b>146</b>	<b>55</b>	<b>6</b>	<b>3</b>	<b>3</b>	<b>10</b>	<b>13</b>	<b>32</b>	<b>25</b>	

\* Not all *V. alginolyticus* isolates are sent to the Consultant Laboratory for Vibrios for analysis and figures therefore do not reflect actual numbers detected. Abbreviations: trh, TDH-related haemolysin; tdh, thermostable direct haemolysin; DE, Germany; NL, Netherlands; BD, Bangladesh; EC, Ecuador; IN, India; TH, Thailand; VN, Vietnam; FR, France; NO, Norway; GR, Greece. The number in brackets after the country code indicates the number of samples from the corresponding country.

As a general rule, occurrence of *Vibrio* spp. in seafood products can be attributed to the natural occurrence of these bacteria in the aquatic environment. In most cases, there is no correlation between faecal coliform microbes introduced into bodies of water by anthropogenic factors and *Vibrio* titres in the aquatic environment. On the other hand, a positive correlation between water temperature and *vibrio* concentrations has been demonstrated in several regions around the world. Other environmental factors, such as water salinity, can have either a positive or a negative effect on the occurrence and abundance of vibrios, depending on the *Vibrio* species under consideration. For many *Vibrio* species, the literature describes how these bacteria may enter a dormant state under certain conditions (lack of nutrients, low temperatures, changes in osmotic concentrations, etc.), which makes direct re-culturing on suitable growth media more difficult. This dormant state is also described as viable-but-non-culturable (VBNC) (Oliver, 2010). The effective revitalisation of such bacteria can typically be achieved by the use of suitable media and an increase in temperature (temperature upshift) (Oliver, 2010). Such conditions should be present when utilising the horizontal ISO method 21872-1 for the detection of enteropathogenic vibrios in food by means of the mandatory two-stage (primary, secondary) enrichment.

### 3.1.2 Hazard characterisation

#### 3.1.2.1 Vibrios as pathogens in food

##### *V. parahaemolyticus*

*V. parahaemolyticus* is well known as one of the primary causes of seafood-borne gastroenteritis in conjunction with the consumption of raw or insufficiently well-cooked products (FAO/WHO, 2011; 2020). Infections are self-limiting and of average severity. Symptoms include diarrhoea with abdominal cramps, nausea, vomiting, headaches and mild fever, and typically last for three days on average in patients with a healthy immune system (Nair et al., 2007). Pathogenic *V. parahaemolyticus* strains are responsible for most seafood-associated infections in the United States, many Asian countries (FAO/WHO, 2011) and South America (Velazquez-Roman et al., 2014).

Two pandemic clones of *V. parahaemolyticus* have caused outbreaks of illness on several continents. The O3:K6 clone with the sequence type ST3 was first discovered in India, and spread during the period 1995 to 2005 within Southeast Asia, the Americas and Africa, also reaching Europe (Spain and France). Another pandemic clone with the sequence type ST36 (known as the Pacific Northwest clone) was responsible for outbreaks in the Americas (North and South America) and in Spain during the period 2012 to 2016 (Martinez-Urtaza and Baker-Austin, 2020).

Compared with Asia and the USA, *V. parahaemolyticus* infections have been reported only rarely in Europe to date. This may be attributable to a low incidence rate or may simply result from a lack of epidemiological surveillance programmes for *Vibrio*-associated illnesses. The pathogenicity of *V. parahaemolyticus* is primarily correlated with the possession of genes that code for haemolysins (TDH = thermostable direct haemolysin and/or TRH = TDH-related haemolysin) (Nishibuchi and Kaper, 1995; Park et al., 2000). Both epidemiological studies and animal experiments have indicated that at least one type 3 secretion system (T3SS2) also has an important role in the pathogenicity of *V. parahaemolyticus* (Park et al., 2004). This T3SS2 is strongly correlated with the presence of *tdh* and/or *trh* genes, and is further subdivided into T3SS2 $\alpha$ , which is associated with the *tdh* gene on a pathogenicity island, and T3SS2 $\beta$ , which is located on a different pathogenicity island in the vicinity of the *trh* gene (Park et al., 2004). In addition, the *trh* gene is genetically associated with a urease gene cluster. Most *V. parahaemolyticus* strains do not possess the *tdh* and/or the *trh* gene(s), however, and are viewed as environmental strains. The results of epidemiological investigations

in Japan indicate that *tdh* gene-bearing strains are more likely to cause gastrointestinal infections than *trh*-positive strains (Saito et al., 2015). Roughly 90 % of all *V. parahaemolyticus* infections during the period under investigation were caused by *tdh* gene-bearing strains without the *trh* gene, although *trh*-positive strains were more commonly detected in oysters and mussels.

In Germany, *tdh*-positive *V. parahaemolyticus* strains have been found in imported seafood only very rarely to date (investigations conducted by the BfR's Consultant Laboratory for Vibrios). There have been no reports of such strains occurring in German coastal waters so far (Huehn et al., 2014). In contrast, *trh*-positive *V. parahaemolyticus* isolates do occur in these areas, although rarely (Bechlars et al., 2015). In general, *trh* gene-bearing strains are detected in a range of roughly 3 % to 5 % in North European coastal areas (Hervio-Heath et al., 2002; Ellingsen et al., 2008), with occurrence tending to rise slightly in French coastal areas (Robert-Pillot et al., 2004; Bechlars et al., 2015). Despite the occurrence of such strains in German waters and their detection in oysters and common mussels from German production regions, no intestinal infections resulting from *V. parahaemolyticus* strains have been documented in Germany to date.

*V. parahaemolyticus* is sensitive to stomach acid (Yeung and Boor, 2004a) and older sources report that the infectious dose ranges from  $10^7$  to  $10^8$  cells (Yeung and Boor, 2004b). This value is also currently stated as the infectious dose by the Canadian health authorities (Government of Canada, 2011). However, it is not clear whether the infectious dose may be lower for the more virulent pandemic clones (Yeung and Boor, 2004b).

### *V. cholerae*

The gram-negative bacterium *V. cholerae* is a species that forms part of the normal flora of aquatic ecosystems worldwide. Since *V. cholerae* bacteria can also be grown on culture media without sodium chloride (NaCl), they may also be present not only in saline marine waters but also in inland fresh waters such as rivers and lakes, where salinity is very low (Kirschner et al., 2018; Vezzulli et al., 2020). On account of its large number of surface antigens, the species can be subdivided into more than 200 serogroups (Vezzulli et al., 2020). Strains of *V. cholerae* belonging to the serogroups O1 and O139 are the cause of cholera, an epidemic disease whose symptoms include diarrhoea. Cholera epidemics are largely limited to countries with inadequate public hygiene systems and occur as a result of limitations in public sanitary systems, which may also follow in the aftermath of natural disasters or political crises. The excretions of sick individuals contain large quantities of *V. cholerae* bacteria, which can enter and contaminate bodies of water. In epidemic regions, an infection is usually the result of an individual being exposed to contaminated drinking water or food that has come into contact with contaminated water (Harris et al., 2012). Cholera is an acute intestinal infection with a short incubation period ranging from less than 24 hours to 5 days. The toxigenic *V. cholerae* strains O1 and O139 produce the cholera toxin, which causes severe watery diarrhoea. *V. cholerae* bacteria colonise and propagate within the intestinal mucosa. This leads to constant vomiting and diarrhoea. This uninterrupted loss of water causes severe bodily dehydration and the loss of vital minerals. Without treatment, 30–50 % of all severe cases of cholera end with the death of the patient within six days. The most important virulence factors from toxic *V. cholerae* include cholera toxin (CT) and a type IV pilus (Zuckerman et al., 2007). Humans are the sole host for cholera bacteria from the O1/O139 serogroups. Cholera infections are rare in Europe. Cases result from individuals having recently visited countries in which the disease is endemic.

The other serogroups of *V. cholerae*, which number over 200, are widespread in the aquatic environment worldwide. Although some strains from these serogroups, referred to collectively as *V. cholerae* non-O1, non-O139, can cause diseases having diarrhoea as a symptom

(Schirmeister et al., 2014), they do not have the capability to cause epidemic outbreaks (Vezzulli et al., 2020). Since most of the non-O1, non-O139 strains do not possess the primary virulence factors of toxigenic *V. cholerae*, they can be distinguished from these by PCR detection methods that target the cholera toxin gene. A series of accessory virulence factors that are also present in toxigenic strains can be found in some non-O1, non-O139 strains. These strains are capable of producing toxins such as haemolysins, RTX toxins, type 3 secretion systems and cholix toxins, but generally cause only self-resolving cases of gastroenteritis (Awasthi et al., 2013; Schwartz et al., 2019). There are a number of cholera toxin-forming serogroups (O141, O75, O37, O10, O12, O6 and O14) without pandemic potential. While these have also been associated with cholera-like illnesses, these have proved to be less severe than true cholera disease (Tobin-D'Angelo et al., 2008; Vezzulli et al., 2020). Since research is still lacking on the precise nature of the virulence factors of these bacteria that are responsible for human infections, there are currently few diagnostic options for reliably distinguishing pathogenic strains from true environmental strains.

Within Germany, gastrointestinal infections by *V. cholerae* non-O1, non-O139 have been reported mostly in conjunction with travel-related illnesses. In some cases of patients complaining of diarrhoea symptoms after returning from regions where cholera is endemic, *V. cholerae* non-O1, non-O139 was subsequently detected in the laboratory (Schirmeister et al., 2014).

#### *V. vulnificus*

*V. vulnificus* is a dangerous bacterial pathogen that can be found in coastal waters worldwide – with a preference for waters having moderate saline levels. The pathogen can cause severe and potentially fatal wound infections (Blake et al., 1979; Lee et al., 2014; Huang et al., 2016; Dupont et al., 2020), and is also responsible for fatalities caused by consumption of contaminated seafood. In the USA, oysters contaminated with *V. vulnificus* are commonly held responsible for fatal cases of infection (FAO/WHO, 2004; Haq and Dayal, 2005; Daniels, 2011). The severity of disease progression is substantially affected by individual health conditions of persons exposed to the pathogen. High-risk individuals include those with a weakened immune system and those with systemic illnesses – particularly chronic liver disease (Bross et al., 2007). In the event of primary septicaemia following consumption of contaminated seafood, the case fatality rate exceeds 50 % (Jones and Oliver, 2009). For people with pre-existing medical conditions, the infectious dose is suspected to be low, at around  $10^3$  CFUs (Jackson et al., 1997; Stavric and Buchanan, 1997; Cruz et al., 2016). Pathogen proliferation is known to be favoured by environmental factors such as warmer water and moderate salinity. Accordingly, the rise in ocean temperatures resulting from climate change has heightened concerns that the number of infections caused by *V. vulnificus* will increase (Baker-Austin et al., 2013). Despite ubiquity of the pathogen in the environment, the number of cases reported is relatively low – an indication of varying virulence between strains in the species. *V. vulnificus* exhibits a high level of genetic diversity and comprises strains with varying degrees of virulence potential (Jones and Oliver, 2009). While most strains are virulent in animal models (Thiaville et al., 2011), several investigations have revealed genetic divergence between strains of clinical origin and environmental strains. Several studies developed methods to distinguish between clinical strains (type C) and environmental strains (type E). The potential virulence markers are based on variations in the sequence of the small subunit of the 16S rRNA gene, the virulence-correlated gene (*vcg*) (Sanjuan et al., 2009) and the *pilF* gene, which codes for a protein that is required for type IV pilus assembly (Baker-Austin et al., 2012). To date, however, there are no reliable genetic markers available to define individual strains as apathogenic environmental strains. In Germany, the strains, which have caused severe and fatal wound infections identified, are primarily type E (Bier et al., 2013).

In Germany, *V. vulnificus* bacteria are found in low-salinity marine ecosystems such as the Baltic Sea or deltas of large rivers that flow into the North Sea (Boer et al., 2013; Huehn et al., 2014). Since this marine environment exhibits a faster rate of warming for surface water temperatures, especially in summer, *V. vulnificus* wound infections in humans following contact with seawater are more numerous in years having long periods of warm weather. Locally acquired foodborne infections have not been reported in Germany to date. Analyses have shown that *V. vulnificus* does occur in imported seafood but is an uncommon finding (see table 1).

#### *V. alginolyticus*

The species *V. alginolyticus* is frequently detected in seafood (Lhafi and Kühne, 2007; Huehn et al., 2014; Vu et al., 2018b), since this species is widespread in oceans and tolerates high levels of salinity (Boer et al., 2013). Gastrointestinal infections are rare, while wound and ear infections caused by *V. alginolyticus* after contact with seawater are described more frequently in the literature (Baker-Austin et al., 2020). In the USA, this species is the *Vibrio* species that is most frequently associated with wound and ear infections (Morris, 2019).

#### *V. metschnikovii*

The species *V. metschnikovii* has also been detected frequently in samples of seafood (table 1). A Norwegian investigation looking at fish and mussel samples identified *V. metschnikovii* as the secondmost common *Vibrio* species after *V. alginolyticus* (Hakonsholm et al., 2020). In terms of taxonomy, *V. metschnikovii* was defined as a novel species in the *Vibrio* genus in the 1970s. This species is distinguished from other *Vibrio* species by its inability to form cytochrome oxidase and to reduce nitrate (Farmer III and Janda, 2004). *V. metschnikovii* is a natural inhabitant of the aquatic environment, and has been isolated from seawater, river deltas and effluent, as well as from fish, crustaceans, oysters and sick birds (Ceccarelli et al., 2019). In particular, occurrence was documented as widespread in rural communities in Brazil, Nigeria, Peru and South Africa. This species is not well-characterised to date. The literature cites *V. metschnikovii* bacteria in relation to cases of extra-intestinal illness, wound infections, pneumonia and cardiovascular diseases, but also in cases having diarrhoea as a symptom (Dalsgaard et al., 1996). Overall, the role of *V. metschnikovii* as a potential human pathogen remains unclear, since these illnesses have been described only as particular case studies with highly variant symptoms (Morris, 2019).

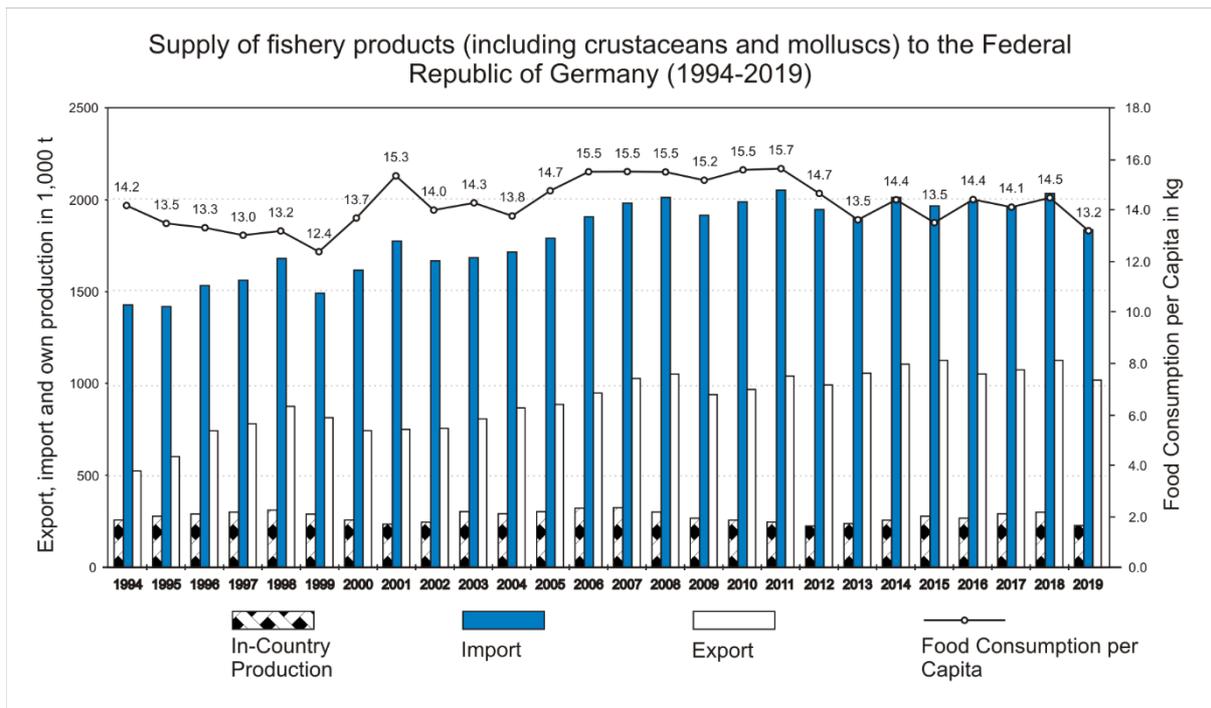
#### Other *Vibrio* species

Table 1 refers to further *Vibrio* species (*V. mimicus*, *V. fluvialis*, *V. furnissii* and *V. harveyi*), which have been described in connection with gastrointestinal infections. When compared with the three potentially enteropathogenic species *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae*, these other species are detected only occasionally in food products in Germany and are therefore not addressed further in the context of this risk assessment.

### 3.1.3 Exposure assessment

On account of their high levels of protein, vitamins and minerals, fish and seafood are considered an important and highly nutritious foodstuff (Institute of Medicine of the National Academy of Sciences, 2007). Demand for these foods has grown both in Europe and worldwide over the last decades. According to the FAO, consumption of fish in the US amounted to no less than 156.2 million tonnes in 2019. This is equivalent to an annual consumption of

roughly 20 kg per capita. These values are lower for Germany. The Federal Office for Agriculture and Food (BLE) gives a figure of 13.2 kg per capita for consumption in 2019 (fig. 1: chart of per-capita consumption). Alongside products from fish or shellfish caught in the wild from rivers, lakes and oceans, products from aquaculture have become significantly more important: in 2014, the contribution from the aquaculture sector for human consumption exceeded the total annual wild catch for the first time. At 21 %, however, German domestic production is currently making only a minor contribution to the total volume of products consumed in Germany.



**Figure 1:** Supply of fishery products (including crustaceans and shellfish) to the Federal Republic of Germany from 1994 to 2019 (source: BLE -532- (2019 provisional))

Gastrointestinal *Vibrio* infections can often be attributed to consumption of raw or insufficiently cooked mussels and crustaceans (Daniels and Shafaie, 2000; Bisha et al., 2012). In the USA, infections with *V. vulnificus* are the leading cause for foodborne fatalities (Daniels, 2011). In Germany, only isolated cases of *Vibrio* infections have been reported in recent years. As ocean surface temperatures rise as a result of climate change, this could lead to an increased occurrence of vibrios in aquatic environments, accompanied by their more extensive colonisation in other local aquatic organisms (Martinez-Urtaza et al., 2010; Vezzulli et al., 2012). This phenomenon has already been described for the Baltic Sea (Frank et al., 2006; Baker-Austin et al., 2010).

### 3.1.3.1. Vibrios in mussels

Annual sales volumes of mussels in Germany amount to 2,000–3,000 t. Most domestic production is concentrated along the coasts of Lower Saxony and Schleswig-Holstein, as well as the island of Sylt as Germany’s sole oyster-producing region. Since 2014, common mussels cultivation has been reintroduced in the Baltic Sea (Kieler Förde), with increasing success

(Schleswig-Holstein Chamber of Agriculture, 2020). Only a few investigations of vibrio contamination in mussels from German fishing grounds have since been made. One detailed study by Lhafi and Kühne was published in 2007 (Lhafi and Kühne, 2007). This study investigated common mussels from various cultivation regions across the German Wadden Sea (Lower Saxony) over a period of one year. Vibrios were detected in 74.4 % of the mussels analysed. Of these, 51.2 % were assigned to the species *V. alginolyticus*. A total of 39.5 % of the vibrios were identified as *V. parahaemolyticus*, although the virulence factors TDH and TRH were not detected. The authors also determined that the number of mussels colonised by *V. parahaemolyticus* decreased with lower water temperatures. A similar phenomenon was observed for the occurrence of *V. vulnificus*, which made up 3.5 % of *Vibrio* isolates. These were only detected at water temperatures between 15 and 20 °C. In addition, 4.7 % of the isolates investigated were assigned to the species *V. cholerae*. However, no virulence-associated genes or serogroups with pandemic potential (O1, O139) were detected (Lhafi and Kühne, 2007). In the course of a systematic review examining the occurrence of potentially pathogenic vibrios in Germany (Huehn et al., 2014), research findings from the Lower Saxony State Office of Consumer Protection and Food Safety (LAVES) – the authority responsible for monitoring the primary production of common mussels in Lower Saxony's Wadden Sea – were also presented. The species most frequently identified in common mussels was *V. alginolyticus*, followed by *V. parahaemolyticus*. Non-O1, non-O139 *V. cholerae* strains were detected in some samples and *V. vulnificus* was rarely present. In 2013, *Vibrio* spp. were detected in all mussels analysed (100 %) and in 87 % of mussels analysed in 2012. In addition, more than one *Vibrio* species was often detected in a single sample and a seasonality of occurrence was also observed. While *V. alginolyticus* was present all year round, the three other species were only detected during the summer and autumn (Huehn et al., 2014). The majority of *V. parahaemolyticus* isolates, which were found in some 40 % of mussels, were probably non-pathogenic environmental strains, since only 1 % of isolates bore the *trh* gene considered to be a primary virulence marker (Nair et al., 2007). No comparable data are available for the Baltic Sea, since this area has seen very little commercial mussel production to date.

The majority of fresh mussels produced in Germany is distributed to wholesalers via the Dutch Mussel Auction in Yerseke (Netherlands). As a result, it is possible that mussels from Lower Saxony are subsequently imported back into Germany via the Netherlands. One exception to this are mussels whose low meat content results in them being shipped out for further processing to mussel cooking plants based in Schleswig-Holstein or the Netherlands. On the other hand, oysters produced on Sylt are sold directly by the producers (Producer Organisation of Schleswig-Holstein Mussel Farmers, 2012). The extent of vibrio contamination in oysters produced here has not been described in the literature to date. Every year, however, the Consultant Laboratory for Vibrios receives 10 to 20 isolates that have been isolated from this product group (see table 1). For the most part, these are strains from the species *V. parahaemolyticus*. In the years 2017, 2019 and 2020, the virulence-associated gene *trh* was detected in some of these isolates.

Around 80 % of the mussels supplied to the EU are sourced from European production. Apart from Germany, common mussels are also cultivated in Denmark, Ireland, Netherlands and the UK. Other European mussel producers include France, Italy and Spain. These last three mostly sell to the European market. Pathogenic vibrios have also been detected in seafood from these countries. In 2014, Robert-Pillot et al. showed that pathogenic *V. parahaemolyticus* (*tdh*- or *trh*-positive) have been detected in 25 % of fish and seafood products investigated (Robert-Pillot et al., 2014). Prevalence figures occasionally exceeding 10 % have also been identified for Italian and Spanish mussels, fish and oysters (Roque et al., 2009; Serracca et al., 2011).

### 3.1.3.2. Vibrios in North Sea (brown) shrimp

Shrimp fishing is an important industry within German domestic production. The brown shrimp (common or North Sea shrimp) is one of the few species of commercially important cold-water shrimp. Although the shrimp has a wide range, extending from the White to the Black Sea, sizeable fishing operations are found only in the North Sea (Wadden Sea) (Schleswig-Holstein Chamber of Agriculture, 2020). To maintain the high quality of the catch, it is processed on-board immediately. The shrimp are cooked in seawater, chilled, brought ashore after no more than 72 hours and distributed to the shrimp grading plants. In 2018, the shrimp fishing industry produced 6,937 t of shrimp. Up to this point in time, however, no investigations of the occurrence of vibrios in German brown shrimp were yet available. Between 6 (2019) and 64 (2018) isolates were also sent annually from this product group to the Consultant Laboratory for Vibrios by the state agencies for consumer and health protection (LUAs) (see table 1). In 21 % to 36 % of cases, *V. parahaemolyticus* was identified, although without the virulence-associated gene *trh* or *tdh*. In 2017, one case of *V. vulnificus* was also detected.

### 3.1.3.3. Vibrios in imported seafood

The majority of seafood consumed in Germany is imported from countries in which pathogenic vibrios are endemic. These countries are typically characterised by having warm water temperatures, which offer optimum conditions for the proliferation of vibrios. As water temperatures rise, this is accompanied not only by an increase in vibrio populations but also by changes in the proportions of species present (Huehn et al., 2014). While the occurrence of pathogenic vibrios with corresponding virulence factors is currently relatively low in the Baltic and North Sea, vibrios with virulent properties are now increasingly detected in regions whose water temperatures remain uniformly warm (DePaola et al., 2003; Flynn et al., 2019). This is also reflected by the occurrence of these bacteria in food (Elhadi et al., 2004; Ottaviani et al., 2013). A study by Stöppelmann and Fieseler (Stöppelmann and Fieseler, 2020) compared data from the literature on the occurrence of *V. parahaemolyticus* and *V. vulnificus* in various categories of seafood caught around the world. Strikingly, both *V. parahaemolyticus* isolates in general and those isolates with human pathogenic potential (*tdh*, *trh*) were detected particularly frequently in seafood from warmer oceanic regions in North, Central and South America as well as Asia (especially in China and India). In comparison, the prevalence of *V. vulnificus* in European foods of marine origin is significantly lower. In their literature review, Stöppelmann and Fieseler stated an average prevalence of 17 % in the fish and seafood investigated (Stöppelmann and Fieseler, 2020). However, the number of studies included was significantly lower. *V. vulnificus* was frequently isolated especially from oysters (34.2 %), prawns and shrimp (14.9 %), fish (14.1 %) and mussels (2.5 %). Following trend can be seen here, namely that the prevalence of *V. vulnificus* in Europe (<20 %) appears to be lower than in China and the USA, where this pathogen has sometimes been detected in more than 50 % of seafood samples analysed (Cook et al., 2002; Chen et al., 2010; Ji et al., 2011; Johnson et al., 2012). Among these studies, there are also a few investigations conducted in Germany, which address food products available in the domestic retail trade (Lehmacher and Hansen, 2007; Mitzscherling and Kühne, 2008; Messelhäusser et al., 2010; Vu et al., 2018b). While these foods are often imported from neighbouring European countries such as Denmark, France, Ireland, Italy, Netherlands, Norway or Spain, they may also be of international origin. Important countries from which seafood is imported are found both in the Americas (Chile, Ecuador, Honduras, Peru, USA) as well as in Asia (Bangladesh, China, India, Indonesia, Philippines, Thailand, Vietnam) and Oceania (New Zealand). To our

knowledge samples were investigated from the retail trade in Berlin and Bavaria. In all German studies, the distribution of the species detected was confirmed in the same way as detected in other countries. Alongside *V. alginolyticus*, highest prevalence figures were found for *V. parahaemolyticus* (5.21 % (Lehmacher and Hansen, 2007) to 27.5 % (Vu et al., 2018b)), followed by *V. cholerae* (6.3 %) (Vu et al., 2018b) and *V. vulnificus* (0.6 %) (Vu et al., 2018b). With the exception of one isolate (*V. parahaemolyticus* with *trh2*), no virulence-associated genes were detected in any of the food samples investigated. This analyses also showed that no pathogenic vibrios had been isolated from previously cooked samples (Messelhäusser et al., 2010) and that unpeeled samples (whole with shell: 96.6 %) were contaminated with vibrios significantly more often than peeled samples (without head: 60 %) (Mitzscherling and Kühne, 2008).

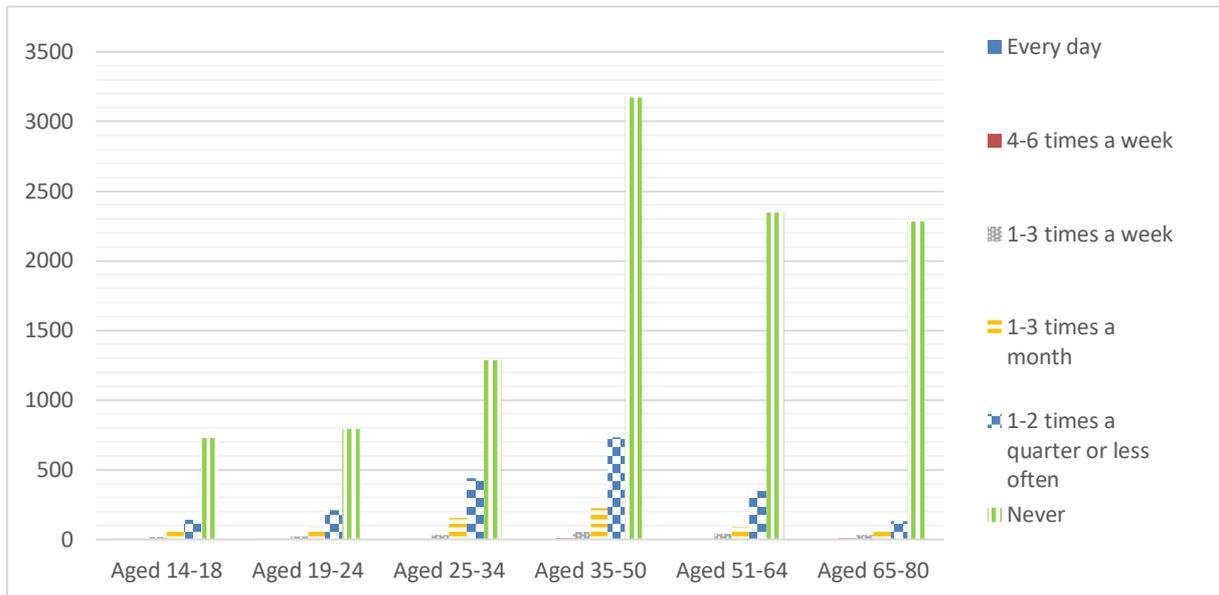
#### 3.1.3.4 Dietary habits in Germany

A study that has since become the representative study on dietary habits in the German population was carried out between 2005 and 2006 (National Nutrition Survey II (NVS II)). This study also contains data on the consumption of saltwater and freshwater fish, as well as shellfish and crustaceans. Taking into consideration three separate survey methods (dietary history, 24-hour recall, weighing log), data on the dietary habits of 13,926 individuals aged between 14 and 80 were ultimately collected throughout Germany (Krems et al., 2006; MRI, 2008). Overall, 19 % of respondents stated that they had consumed saltwater fish during the survey period. The consumption of freshwater fish, shellfish and crustaceans amounted to a proportion between 0.4 % and 1.8 %. The expected regional differences in consumption – with maximum quantities along Germany's coasts – were not confirmed. While people in Hamburg consumed fish the most, women from Mecklenburg-Western Pomerania were the population group with the lowest quantities consumed of fish and seafood. Both men and women from Bavaria also achieved mid-table rankings in terms of the quantities of fish and fishery products they consumed, despite living the furthest away from the sea in Germany. This blurring of the North-South gap suggests increasingly homogenous flows of goods within Germany.

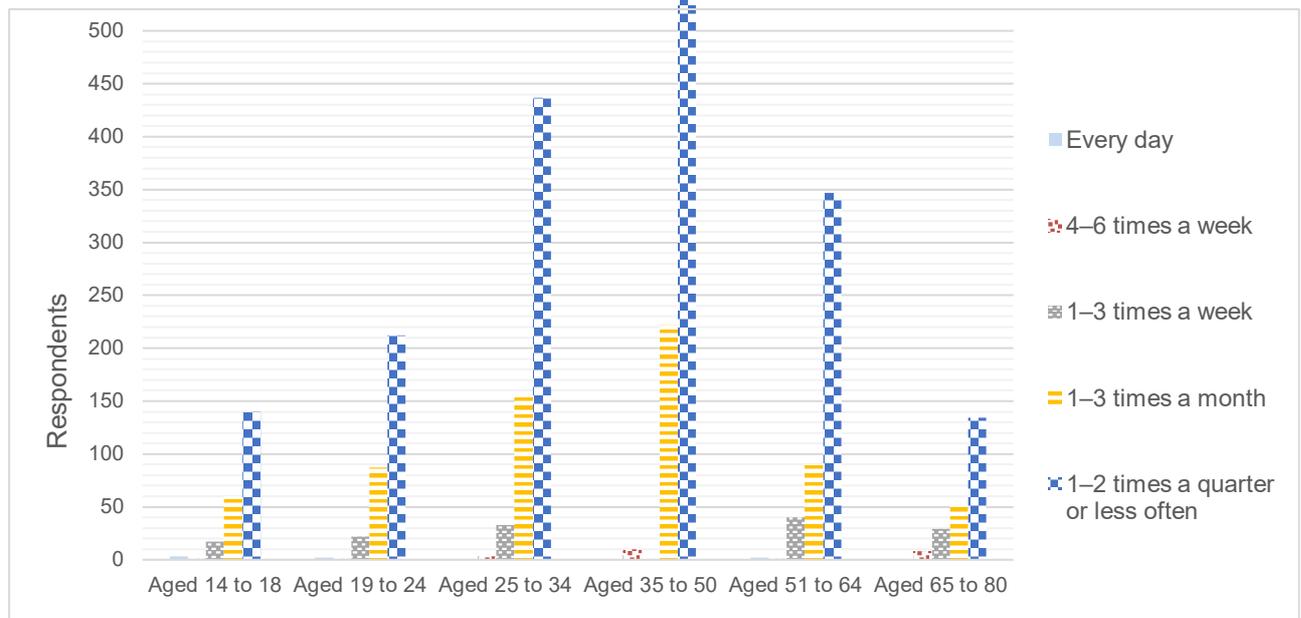
The consumption data were also analysed in terms of short-term and long-term intake. For short-term consumption based on body weight (BW), similar figures were observed for saltwater/freshwater fish and shellfish (3.9 to 4.7 g/kg BW/day), while consumers of crustaceans consumed 2.6 g/kg BW on a daily basis. In a gender-based comparison, men and women consumed roughly equal quantities of saltwater fish. However, women consume almost double the quantities of freshwater fish. This situation is reversed in the case of shellfish and crustaceans. In terms of long-term consumption, saltwater and freshwater fish are consumed by men and women in roughly equal quantities (0.8 and 1.0 g/kg BW/day), while lower quantities of shellfish and crustaceans are consumed by both genders (0.4 and 0.2 g/kg BW/day). In terms of average consumption, men and women do not differ in either of the groups considered. When considering the frequent seafood consumer group, however, a picture similar to that of short-term consumption emerges. Women consume significantly greater quantities of freshwater fish, while men consume greater quantities of shellfish and crustaceans.

In consideration of the generally elevated bioburden found in raw fish and seafood products, the consumption data were also analysed in terms of this characteristic (figure 2). The data revealed that the majority (75 %) of all respondents never consume products from this category. In comparison, the number of individuals who consume raw fish or seafood products is inversely proportional to the frequency of consumption (figure 3) (one or two times/month: 5 %; daily: 0.1 %). Figure 3 also shows an age-based trend, with a rising frequency of consumption proportional to higher age, reaching a maximum for individuals aged between 35

and 50. In older individuals, the quantity of fish and seafood consumed raw then declines once again.



**Figure 2:** Frequency of consumption of raw fish/raw mussels, by age group (basis: NVS II, survey form)



**Figure 3:** Excerpt from figure 2, 'Frequency of consumption of raw fish/raw mussels, by age group', without the response 'Never' (basis: NVS II, survey form)

The underlying data from the consumption study exhibit a number of uncertainties. They stem from a study that was conducted 15 years ago and the data should therefore be re-verified. In addition, the available dataset from a breakdown by age group is too small to be able to draw representative conclusions. It can however offer indications about German dietary habits. While the risk of an infection with vibrios can be categorised as low, owing to a low rate of exposure and adequate heating (maintaining a core temperature of 70 °C for two minutes), there remains a risk of cross contamination during handling or from the consumption of raw food products.

### 3.1.4 Risk characterisation

#### 3.1.4.1. Affected population or population group

A hazard from food results primarily from raw and insufficiently heated food products. Mussels and oysters, which are sedentary organisms that feed by filtering the seawater in their environment, may contain higher concentrations of bacteria. In bodies of salt water, vibrios are to be expected as a normal member of the natural bacterial flora and therefore present in bivalve shellfish, where they may even be found in high concentrations. The consumption of live mussels/oysters – especially if contaminated with *V. vulnificus* – can therefore present a hazard, especially for immunosuppressed individuals or those with existing medical conditions, such as chronic liver disease.

Seafood is consumed by all population groups, including higher-risk population groups. Most products available have either been heated or treated using other methods (marinating, smoking, drying, salting, etc.) and should therefore contain lower levels of bacteria. Vibriosis following the consumption of seafood has not been reported in Germany to date. The competent health body, the Robert Koch Institute, notes on its website that the Institute has been aware of only isolated cases of gastrointestinal infections by non-cholera vibrios since 2000.

#### 3.1.4.2. Route and probability of exposure

The transmission of pathogenic vibrios occurs during the consumption of contaminated food (alimentary transmission) that is raw or has been insufficiently heated. Naturally contaminated food products include products in which environmental strains are persistent. Exposure can also occur from consumption of food products and meals that have come into contact with contaminated seafood products via cross contamination, and which are then consumed immediately afterwards. An infectious dose for humans has been given for *V. parahaemolyticus* as roughly  $10^7$  to  $10^8$  colony-forming units (CFUs). The dose may well be lower for the vulnerable population groups mentioned above.

Another route of exposure is presented by open wounds that come into contact with pathogenic vibrios.

#### 3.1.4.3. Probability of occurrence of impairments to health at certain levels of exposure

Reliable data on the probability of occurrence of health impairments following exposure are not available to date. However, it can be assumed that the risk of an impairment to health is correlated with the quantity of pathogenic vibrios consumed and is dependent also on the particular species that is consumed. In addition, the probability of occurrence of an impairment to health should also be assumed to be higher for vulnerable groups. Such groups include individuals whose immune system is not yet fully developed (young children), individuals with a weakened immune system and individuals with chronic organ conditions. Pregnant women are also considered to be a vulnerable group in this context.

#### 3.1.4.4. Frequency of impairments to health

Vibrios are widely prevalent, specifically in food from aquatic sources. The frequency of an impairment to health following the consumption of these products however depends on the particular species consumed. Alongside *V. alginolyticus*, which has previously been only very rarely associated with gastrointestinal illness, *V. parahaemolyticus* is a frequently isolated species. The pathogenicity of this species is associated with the presence of two potential virulence factors (TDH, TRH). However, the genes that code for these factors have been detected only rarely in the isolates obtained in Germany. While bacteria of the species *V. cholerae* may be detected in domestic food products, they do not belong to the serogroups that cause classic cholera. To date, gastrointestinal infections with other serogroups (non-O1/non-O139) have also been described mostly in connection with travel-related illnesses. In contrast, the species *V. vulnificus* has previously been isolated at only low percentages from food sourced from the German retail market. Research on the factors that influence the pathogenicity of an isolate also remains inconclusive. However, this pathogen has the potential to impair the health of patients from the corresponding risk group to a considerable degree. Foodborne infections caused by vibrios have not been documented in Germany to date. However, it should be remembered that such infections have been notifiable only since 2020. As a result, potential cases occurring before this period may not have been recorded and the real figure is therefore likely to be higher. As a result of the minimal level of exposure, however, the probability of an impairment to health is still considered to be low. Though, a change to this classification as a result of changing climatic conditions over the next few decades cannot be ruled out.

#### 3.1.4.5. Type, duration, reversibility and severity of impairments to health

The type and manifestation of an impairment to health resulting from an infection with vibrios is also dependent on the individual species. Cases of illness caused by *V. parahaemolyticus* characteristically include diarrhoea with abdominal cramps, nausea, vomiting, headaches and mild fever, and typically last for three days on average in patients with a healthy immune system. Infections are self-limiting and of average severity.

In cases caused by *V. cholerae*, a distinction must be made between infections with the serogroups O1/O139 and other serogroups (non-O1/non-O139). The first groups are known to cause cholera, an acute intestinal infection with a short incubation period ranging from less than 24 hours to 5 days, associated with watery diarrhoea and constant bouts of vomiting. This uninterrupted loss of water causes severe bodily dehydration and the loss of vital minerals. Without treatment, 30–50 % of all severe cases of cholera end with the death of the patient within six days. Infections with non-O1/non-O139 serogroups typically only result in cases of self-resolving gastroenteritis, although they may cause cholera-like disease in the presence of cholera toxin, yet once again without pandemic potential and with a less severe progression.

Foodborne infections with *V. vulnificus* can also be associated with the occurrence of gastroenteritic symptoms. Primary septicaemia can also develop in individuals with pre-existing medical conditions, especially in the case of people with chronic liver disease. In the event of primary septicaemia following the consumption of contaminated seafood, the case fatality rate is more than 50 %.

#### 3.1.4.6. Evidence for a causal relationship

The relationship between consumption of seafood products contaminated with human pathogenic vibrios and gastrointestinal infections has been proven and is accepted scientific knowledge (CDC, 2019; RKI, 2020).

#### 3.1.4.7. Uncertainties and variabilities

1) No data are available on the quantitative contamination of seafood in Germany by the three enteropathogenic *Vibrio* species. The level of exposure to *Vibrio* bacteria in food products for German consumers is therefore unknown.

2) It can be assumed that adequate heating results in the inactivation of all pathogenic vibrios. Any uncertainty about the risk from excessive vibrio contamination is therefore largely related to those products that are consumed raw or after only brief cooking. Such foods include oysters in particular and some mussels, as well as fish that is consumed raw, such as sashimi or raw seafood ingredients in sushi. Since only isolated cases of gastrointestinal infections resulting from contaminated oysters/mussels have been brought to attention in Germany, it may be assumed that, even for these types of foods, exposure to *Vibrio* bacteria is currently so low that the probability of occurrence of impairments to health after consumption of these products is also low.

3) Uncertainty arising from lack of reliable data on quantitative contamination of oysters and mussels by vibrios is considered to be low, owing to the low rates of infection. However, it should also be remembered that vibrio infections have only been a notifiable disease since 2020. This lack of a notifiable status also means that very few investigations of enteropathogenic vibrios were conducted in patients presenting with diarrhoea as a symptom in Germany. Potential cases of *Vibrio* infection therefore went unrecorded, especially since these

bacteria require highly specific culture methods for detection. Quite possibly, genuine cases of gastrointestinal *Vibrio* infection have not been recorded.

4) A quantitative characterisation of enteropathogenic vibrios in oysters/mussels would be one approach suitable for determining uncertainty about health risks. For characterisations of this type, culture protocols (MPN method) or real-time PCR methods could be applied in order to detect enteropathogenic vibrios.

#### 3.1.4.8. Further research needed

Further research is required to address the following questions:

- Optimisation and standardisation of methods for quantifying vibrios in food products  
A standardisation of quantitative methods for detection of vibrios is needed in order to be able to compare prevalence figures. In particular, real-time PCR methods should be validated here in terms of their suitability. Culturing methods, e.g. using MPN, are very time-consuming and typically require further analyses in order to identify the species.
- Prevalence figures for vibrios in live mussels/oysters  
How high is the *Vibrio* titre in German mussels/oysters at harvest and how does this change if the cold chain is maintained? Does this titre decline over longer periods of refrigeration?
- Prevalence figures for vibrios in ready-to-eat food products such as sushi or algae/algae-products  
Do cases of *Vibrio* contamination occur in these food products and does this give rise to a public health concern?
- Verification of the effectiveness of technologies to reduce vibrio contamination in food products  
Experimental studies show that treatment of food with physical procedures such as moderate heat treatment (up to 50 °C) or applying pressure methods have the capability to reduce *Vibrio* concentrations. These procedures could also be applied in the case of food products for which higher vibrio concentrations have been determined.
- Research on the virulence factors of the three enteropathogenic *Vibrio* species that play a role as the cause of gastrointestinal infections  
In the case of *V. vulnificus*, reliable markers that would enable a distinction between pathogenic and environmental isolates are still absent. For *V. parahaemolyticus*, the role of the type 3 secretion system in gastrointestinal infections should be clarified and the significance of TRH haemolysins for such illnesses should be identified. In the case of *V. cholerae* non-O1, non-O139 isolates, research is also needed on the relevant virulence factors for gastrointestinal infections, so as to be able to identify suitable markers for the detection of pathogenic isolates in food production.

#### 3.1.4.9. Controllability of the risk

To guard against foodborne vibrio infections, the BfR recommends consumers to take the following steps:

- Strict compliance with general rules of hygiene on the handling of food, paying particular attention to refrigeration, separate storage and preparation of food products, in order to avoid cross contamination with other foods.

- Following instructions given on products about the adequate heating of the food before consumption (interior temperatures of at least 70 °C for two minutes). Corresponding instructions should be clearly printed on packaging and also provided to consumers purchasing goods in bulk.
- Immediate consumption or preparation of food after removal from the refrigerator. Consumers should consume seafood products within two hours of removal from the refrigerator, to minimise the time available for the proliferation of any vibrio bacteria that may be present. This guidance applies in particular for ready-to-eat food products.
- Wearing gloves to reduce skin contact. Instructions should be provided stating that vibrios can enter the human body during the process of peeling or deveining/gutting shrimp and other raw, unprocessed seafood products via small, unnoticed injuries to the skin or patches of damaged skin, where they can then cause wound infections that may in some circumstances progress seriously.

### 3.2 Risk management options, recommended measures

In seafood industry facilities that process raw products, gloves should be worn when handling raw goods.

To make sure that personnel understand the reason for this measure, workers should be informed that vibrios can enter the human body via small, unnoticed injuries in the skin or patches of damaged skin, where they can then cause wound infections that may in some circumstances progress seriously.

Since currently there are no EU regulations setting out microbiological limit values for vibrios in seafood, the following recommendations apply for food, both raw and ready-to-eat:

- Absence of pandemic O1, O139 *V. cholerae* strains with cholera toxin (*ctx*) and of *ctx*-positive strains of other serogroups
- Absence of *V. vulnificus*
- Absence of toxin-forming *V. parahaemolyticus* strains (*tdh+*, *trh+*)

Cross contamination between foods should be avoided in the retail trade. The presentation of seafood products on ice in sale counters should be organised in such a way that ice and iced water is not mixed or reused between separate products. Accordingly, storage and presentation of seafood products should be organised with the use of separate containers.

When preparing seafood products, maintaining an interior temperature of 70 °C in the food to be consumed for two minutes is a reliable method to ensure the inactivation of these pathogens.

### 3.3 Other aspects

In this section, questions listed in section 1 are answered in the order given:

1. Which species within the bacterial *Vibrio* genus (non-cholera vibrios) are, in the context of foodborne illnesses, of paramount importance as human disease causative agents for the investigation of food potentially contaminated with pathogenic vibrios (non-cholera vibrios)?

Investigation of foods for potentially pathogenic *Vibrio* species should concentrate on the three potentially enteropathogenic species *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio cholerae*. These three *Vibrio* species are widespread in aquatic ecosystems worldwide, and can therefore be present both in international trade goods and in German food products that are locally produced in coastal waters. Over the course of the last 20 years, the World Health Organisation (WHO) and the Food and Agriculture Organisation of the United Nations (FAO) have published several risk assessments addressing the occurrence of these three species in certain food products and hazards resulting from consumption of contaminated products (see e.g. FAO/WHO, 2010b; 2020). Following these WHO/FAO studies, an international standard was developed that includes a standardised procedure for detecting these three pathogens in food products (International Standard ISO-21871: 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*). *Vibrio parahaemolyticus* is a common causative agent for diarrhoea in many parts of the world, especially Southeast Asia and the Americas, and is very often present in food of marine origin. *Vibrio vulnificus* causes disease only relatively rarely. For persons with a weaker immune system, such as older individuals with pre-existing conditions, infections with *Vibrio vulnificus* can have a very severe progression and may prove fatal. *Vibrio cholerae* is a global species that may occur both in coastal areas and in inland waters. Appropriate food analysis protocols must be capable of confirming the absence of cholera-causative isolates of the *Vibrio cholerae* serogroups O1 and O139. The cholera toxin-bearing strains may be present in international trade goods that are exported from countries in which toxigenic strains are endemic (e.g. shrimps from developing economies). *Vibrio cholerae* isolates that are not members of the toxigenic serogroups (designated as *Vibrio cholerae* non-O1, non-O139) can also produce mild cases of diarrhoea but do not possess pandemic potential but have not been sufficiently characterized for virulence factors to date.

A number of other *Vibrio* species have also been described as causative agents of gastrointestinal infections. Of these, the commonest isolates detected in food products in Germany and sent to the BfR by consumer and health protection agencies were strains from the species *Vibrio alginolyticus* and *Vibrio metschnikovii*. Isolates from *Vibrio alginolyticus* have been detected only very rarely in conjunction with intestinal infections and, while primarily playing a role in soft tissue and ear infections after contact with seawater (Baker-Austin et al., 2018), do not have a status as a foodborne pathogen. They are one of the commonest *Vibrio* bacteria found in ocean waters, which explains their frequent occurrence in investigations of food from this environment. *Vibrio metschnikovii* is also a common component of the autochthonous flora in bodies of water but infections are rare (Baker-Austin et al., 2018).

2. What insights are available to the BfR on the prevalence and importance of pathogenic vibrios (non-cholera vibrios) in seafood products sold in the retail sector, and in mussels and oysters produced in Germany?

On account of their high levels of protein, vitamins and minerals, fish and seafood are considered an important and highly nutritious foodstuff (Institute of Medicine of the National Academy of Sciences, 2007). The consumption of these foods has grown both in Europe and worldwide over the last few decades. For Germany, the Federal Office for Agriculture and Food (BLE) gives a figure of 13.2 kg per capita for consumption in 2019. Alongside products from fish caught in the wild from rivers, lakes and oceans, aquaculture has become significantly more important: in 2014, contribution from this sector for human consumption exceeded the total annual wild catch for the first time. At 21 %, however, German domestic production makes only a minor contribution to the total volume of products consumed in Germany.

Gastrointestinal *Vibrio* infections can often be attributed to the consumption of raw or insufficiently cooked mussels and crustaceans (Daniels and Shafaie, 2000; Bisha et al., 2012). In the USA, infections with *V. vulnificus* are the leading cause of foodborne fatalities (Daniels, 2011). In Germany, only isolated cases of *Vibrio* infections have been reported in recent years. As ocean surface temperatures rise as a result of climate change, however, this could lead to an increased occurrence of vibrios in aquatic environments, accompanied by their more extensive colonisation of other aquatic organisms (Martinez-Urtaza et al., 2010; Vezzulli et al., 2012). This phenomenon has already been described for the Baltic Sea (Frank et al., 2006; Baker-Austin et al., 2010).

Mussel fishing is an important industry within German seafood production. Most domestic production is concentrated along the coasts of Lower Saxony and Schleswig-Holstein, as well as the island of Sylt as Germany's sole oyster-producing region. Since 2014, common mussels have again been cultivated in the Baltic Sea (Kieler Förde), with increasing success (Schleswig-Holstein Chamber of Agriculture, 2020). Only a few investigations have been made of the vibrio contamination of mussels from German fishing grounds. One detailed study on the occurrence of vibrios in a number of mussel cultivation areas in Lower Saxony was published by Lhafi and Kühne in 2007 (Lhafi and Kühne, 2007). In the course of a systematic review of the occurrence of potentially pathogenic vibrios in Germany (Huehn et al., 2014), research findings from the Lower Saxony State Office of Consumer Protection and Food Safety (LAVES) – the body responsible for monitoring the primary production of common mussels in Lower Saxony's Wadden Sea – were also published. Both studies showed that the dominant species was *V. alginolyticus*, followed by *V. parahaemolyticus*. Non-O1, non-O139 *V. cholerae* strains were also detected in some samples, with *V. vulnificus* being more rarely detected. According to LAVES, vibrios were detected in all mussels analysed (100 %) in 2013 and in 87 % of common mussels analysed in 2012. In addition, more than one *Vibrio* species was often isolated from a single sample and a seasonality of occurrence for vibrios was also observed. While *V. alginolyticus* was present all year round, the three other species were only detected during the summer and autumn (Huehn et al., 2014). The majority of *V. parahaemolyticus* isolates, which were detected in some 40 % of mussels, were probably non-pathogenic environmental strains, since only 1 % of isolates bore the *trh* gene considered to be a virulence marker (Nair et al., 2007). Lhafi and Kühne identified no *trh* gene-bearing isolates in their investigations (Lhafi and Kühne, 2007). No comparable data are available for the Baltic Sea, since this has seen very little commercial mussel production to date.

The majority of the fresh mussels produced in Germany are distributed to wholesalers via the Dutch Mussel Auction in Yerseke (Netherlands). As a result, it is possible that mussels from Lower Saxony are subsequently imported back into Germany via the Netherlands. One exception to this are mussels whose low meat content results in them being shipped out for further processing to mussel cooking plants based in Schleswig-Holstein or the Netherlands. On the other hand, oysters produced on Sylt are also sold directly by the producers (Producer Organisation of Schleswig-Holstein Mussel Farmers, 2012). The extent of vibrio contamination in oysters produced here has not been described in the literature. Every year, however, the Consultant Laboratory for Vibrios in Food receives 10 to 20 isolates that have been isolated from this product group (see table 1). For the most part, these are strains from the species *V. parahaemolyticus*, in which the virulence-associated *trh* gene has also been detected in a few cases.

Around 80 % of the mussels supplied to the EU are sourced from European production. Apart from Germany, common mussels are also cultivated in Denmark, Ireland, Netherlands and the UK. Other European mussel producers include France, Italy and Spain. The latter three mostly sell to the European market. Pathogenic vibrios have also been detected in sea-

food from these countries. In 2014, Robert-Pillot et al. showed that pathogenic *V. parahaemolyticus* (*tdh*- or *trh*-positive) have been detected in 25 % of fish and seafood products investigated (Robert-Pillot et al., 2014). Prevalence figures occasionally exceeding 10 % have also been identified for Italian and Spanish mussels, fish and oysters (Roque et al., 2009; Serracca et al., 2011).

The majority of seafood consumed in Germany is imported from countries in which pathogenic vibrios are endemic. These countries are typically characterised by having warm water temperatures, which therefore offer optimum conditions for the proliferation of vibrios. As water temperatures rise, this is accompanied not only by an increase in vibrio populations but also by changes in the proportions of species present (Huehn et al., 2014). While the occurrence of pathogenic vibrios with corresponding virulence factors is currently relatively low in domestic waters, vibrios with virulent properties were increasingly found in regions whose water temperatures remain uniformly warm (DePaola et al., 2003; Flynn et al., 2019); this is also reflected by the occurrence of these bacteria in food (Elhadi et al., 2004; Ottaviani et al., 2013). A study by Stöppelmann and Fieseler (Stöppelmann and Fieseler, 2020) compared data from the literature on the occurrence of *V. parahaemolyticus* and *V. vulnificus* in various categories of seafood caught around the world. Strikingly, both *V. parahaemolyticus* isolates in general and those isolates with human pathogenic potential (*tdh*, *trh*) were detected particularly frequently in seafood from warmer oceanic regions in North, Central and South America as well as Asia (especially in China and India). In comparison, the prevalence of *V. vulnificus* in foods of marine origin is significantly lower. In their literature review, Stöppelmann and Fieseler stated an average prevalence of 17 % in the fish and seafood investigated (Stöppelmann and Fieseler, 2020). However, the number of studies included was significantly lower. *V. vulnificus* was isolated especially frequently from oysters (34.2 %), prawns and shrimp (14.9 %), fish (14.1 %) and mussels (2.5 %). A trend can also be seen here, namely that the prevalence of *V. vulnificus* in Europe (<20 %) appears to be lower than in China and the USA, where this pathogen has sometimes been detected in more than 50 % of seafood samples analysed (Cook et al., 2002; Chen et al., 2010; Ji et al., 2011; Johnson et al., 2012). Among these studies, there are also a few investigations that have been conducted in Germany, which address food products available in the domestic retail trade (Lehmacher and Hansen, 2007; Mitzscherling and Kühne, 2008; Messelhäusser et al., 2010; Vu et al., 2018b). While these foods are often imported from neighbouring European countries such as Denmark, France, Ireland, Italy, Netherlands, Norway or Spain, they may also be of wider international origin. Important countries from which seafood is imported are found both in the Americas (Chile, Ecuador, Honduras, Peru, USA) as well as in Asia (Bangladesh, China, India, Indonesia, Philippines, Thailand, Vietnam) and Oceania (New Zealand). To our knowledge samples were investigated from the retail trade in Berlin and Bavaria. In all German studies, distribution of the species found was confirmed in the same way as detected in other countries. Alongside *V. alginolyticus*, the highest prevalence figures were found for *V. parahaemolyticus* (5.21 % (Lehmacher and Hansen, 2007) to 27.5 % (Vu et al., 2018b)), followed by *V. cholerae* (6.3 %) (Vu et al., 2018b) and *V. vulnificus* (0.6 %) (Vu et al., 2018b). With the exception of one isolate (*V. parahaemolyticus* with *trh2*), no virulence-associated genes were detected in any of the food samples investigated. The analyses also showed that no pathogenic vibrios had been isolated from previously cooked samples (Messelhäusser et al., 2010) and that unpeeled samples (whole with shell: 96.6 %) were contaminated with vibrios significantly more often than peeled samples (without head: 60 %) (Mitzscherling and Kühne, 2008).

A more detailed discussion of the significance of pathogenic vibrios in food products available in Germany can be found in section 3.1.3, 'Exposure assessment'.

3. Which trends can be determined from figures on the prevalence of foodborne illnesses as caused by the occurrence of pathogenic vibrios (non-cholera vibrios) in food?

Infections with non-cholera vibrios are rare in Germany. Most of the data on these kinds of infections are provided by cases of illness resulting from direct contact with seawater in coastal regions. According to the Robert Koch Institute (RKI), the annual number of cases in German coastal areas ranged from 0 to 20 during 2002 to 2019, with infections being significantly more common during the warmer summers of 2003, 2006, 2010, 2018 and 2019 (RKI, 2020).

The majority of these cases involved wound and ear infections. Only isolated cases of gastrointestinal illness were reported to the Robert Koch Institute.

The RKI suggests that *Vibrio* infections may have been underdiagnosed because of their non-notifiable status and that there is therefore a lack of reliable surveillance data. Since 1 March 2020, all infections with human pathogenic *Vibrio* spp. are now indeed notifiable in accordance with the Protection Against Infection Act (IfSG) in cases where an acute infection has been diagnosed. In the first year (2020) following the introduction of this notifiable status, 13 cases were reported to the RKI. From these data and the symptoms described in each case, it can be concluded that these were cases of extra-intestinal illness and not gastrointestinal infections. Currently, no distinction is made in the reporting procedure between wound infections and gastrointestinal infections. However, it would be advisable to introduce this distinction in the reported data in the future.

Owing to the paucity of available data, no predictions relating to the future trend for foodborne vibrio infections for Germany are possible at this time. In contrast to other regions around the world, foodborne *Vibrio* infections have been very rare in Europe to date. A retrospective study of gastrointestinal infections caused by *V. parahaemolyticus* conducted in the UK for the period 2008 to 2018 showed that most of these infections were travel-related (Baker-Austin et al., 2020). A study published by the BfR on *V. cholerae* non-O1, non-O139 isolates dating from 2014 also shows that most of the isolates from gastrointestinal infections (seven of eight) originated in patients returning from foreign travel (Schirmeister et al., 2014). Overall, the number of gastrointestinal infections caused by *Vibrio* spp. is very low and it remains to be seen if infections can be increasingly assessed due to the notifiable status of such infections.

4. What are the parameters that promote occurrence, propagation and transmission of pathogenic vibrios (non-cholera vibrios) in food?

Widely prevalent in the environment worldwide, vibrios are primarily to be found in saline waters and wetlands, and therefore present a potential source of contamination for seafood used in the food industry. This risk is also present for the species known as 'non-cholera vibrios', particularly the strains described in association with non-cholera vibriosis, namely *V. parahaemolyticus*, *V. vulnificus* and, in isolated cases, also *V. cholerae* non-O1, non-O139.

In the environment, water temperature and salinity are the primary positive factors for growth, and may be viewed as the most important natural parameters (Martinez-Urtaza et al., 2008). For certain vibrio strains, growth may already start to accelerate as water temperatures rise above 12 °C (Martinez-Urtaza et al., 2008). These strains also include the species assessed here, which have the potential to be pathogenic in humans. Equally, a low to moderate salinity of 1 to 25 ppt also contributes to promote bacterial growth (Martinez-Urtaza et al., 2008; FAO/WHO, 2020). Acting in concert, water temperature and salinity are therefore the two

most influential factors for the frequency of occurrence of vibrios in the environment (Martinez-Urtaza et al., 2008). An optimum balance between these two factors for growth can often be found in the Gulf of Mexico, for example, or on the European Atlantic coast, with varying degrees of salinity, and in European inland seas, especially in the summer months. Based on these findings, it can therefore be assumed that the rising water temperatures caused by climate change, affecting the fishing and harvesting regions along the European Atlantic coast in particular, including the few German producing regions, will be associated with a higher level of occurrence of pathogenic vibrios in these waters. The situation is different in tropical regions, where large deviations in water temperature or fluctuations in salinity occur only rarely. In these regions, a constant concentration of vibrios is observed, although weather conditions may cause brief fluctuations in temperature or salinity over short periods of time (Parvathi et al., 2004; Deepanjali et al., 2005). Research has also shown that the same vibrio species from different regions may also exhibit differences in virulence. Accordingly, the origin of the seafood may present a vulnerability factor in relation to an infection with vibrios for consumers of these products (FAO/WHO, 2020). Study findings have also suggested a number of other parameters that can lead to an increased prevalence of vibrios. One such parameter is the chlorophyll content of water resulting from the algae present in the ecosystem (Martinez-Urtaza et al., 2008; Deter et al., 2010). Water turbidity has also been named as a potential factor in this context, although the mechanism whereby this leads to an increase in the proliferation of vibrios has not yet been clarified (Zimmerman et al., 2007; Johnson et al., 2010). In addition, the presence or absence of certain vibrio-specific bacteriophages also appears to influence their prevalence (Zabala et al., 2009; Bastías et al., 2010). Although bacteriophages generally possess bactericidal properties, studies have repeatedly shown that the presence of certain kinds of bacteriophages can actually result in the promotion of bacterial growth. In relation to pathogenic vibrios, this unusual behaviour has been reported for the lytic bacteriophage VP93, which was isolated from vibrios in Chilean waters (Bastías et al., 2010; García et al., 2013).

Depending on the concentration of vibrios in water, the bacteria colonise the gastrointestinal tract of filtering bivalve shellfish – as well as crustaceans and fish – to varying degrees; they can then proliferate and persist there for the animal's entire lifespan (Gooch et al., 2002; Fernandez-Piquer et al., 2011).

Although the open-water conditions described above (temperature, salinity) play an important role when determining catch months, these can be partially regulated under cultivation conditions. And these conditions can certainly be monitored. Accordingly, the type and nature of cultivation conditions and the catch/harvesting methods present equally important sets of parameters that, on the one hand, promote the occurrence of pathogenic vibrios in food while, on the other, may also be reduced by control measures (e.g. GMP, GHP, HACCP plans) along the seafood food chain.

Since vibrios are temperature-sensitive pathogens, one of the most important factors for the transmission of pathogenic vibrios to humans is the consumption of raw or insufficiently heated food products. Contact between cooked food and raw seafood can also result in the transmission of pathogens to humans. Unhygienic harvesting methods and a lack of adequate options for refrigeration or freezing immediately post-catch or -harvest can also lead to a significantly higher contamination of seafood products with pathogenic vibrios.

5. Which strategies are considered efficient in terms of minimising the occurrence of pathogenic vibrios (non-cholera vibrios) in food, which relate to the cold chain, and the preservation and decontamination methods utilised during the production process (including processing, transportation and storage), and which can influence the contamination of food with pathogenic vibrios (non-cholera vibrios)?

Minimisation strategies aimed at reducing or inactivating bacteria in foods contaminated with vibrios are generally of a physical nature. One especially important measure in the field of minimisation strategies for vibrios in the seafood product cluster is maintaining the cold chain from the catch or harvest to the retail outlet. While this is, in principle, important for any seafood, it is particularly critical for foods intended to be consumed raw. Maintaining the cold chain is an effective way to reduce or even prevent the proliferation of any vibrios that are potentially present. For the bacterium *V. parahaemolyticus* for example, levels of natural environmental contamination have been given as  $10^2$  to  $10^3$  colony-forming units (CFUs) (Alter et al., 2011). An infectious dose of this pathogen for humans ranges from roughly  $10^7$  to  $10^8$  CFUs (Yeung and Boor, 2004b). Higher values than these can be achieved in the space of only two to three hours after the catch or the harvest, as a result of the pathogen proliferating at storage temperatures from 20 to 35 °C (Alter et al., 2011). Maintaining the cold chain – at temperatures of roughly 4 to 10 °C – offers an effective measure for preventing proliferation above an infectious dose (Limthammahisorn et al., 2009; Alter et al., 2011). As a general principle, adherence to Good Manufacturing Practice (GMP), Good Hygiene Practice (GHP) and Hazard Analysis Critical Control Points (HACCP) practice is also a key requirement for food safety in the seafood sector (FAO/WHO, 2007). In contrast to product refrigeration, deep-freezing products (to –20 °C) directly after the catch or harvest can prevent vibrio proliferation entirely (Food and Drug Administration, 2005b). In addition, the deep freezing process also works to reduce bioburden, with moderate reductions in contamination which is even observable at a temperature of 4 °C. The bioburden continues to be reduced as temperatures fall further and storage duration is increased. At a temperature of –20 °C and a storage duration of 35 days, a reduction in contamination with *V. parahaemolyticus* of two to three log levels was observed (Food and Drug Administration, 2005b). Muntada-Garriga et al. also found that, under laboratory conditions, continued storage of oysters inoculated with *V. vulnificus* ( $10^5$  to  $10^7$  CFU/g) at –18 °C and –24 °C for 28 and 15 weeks led to a reduction of microbes below the limit of detection, which the authors described as ‘complete inactivation’ (Muntada-Garriga et al., 1995). Another study conducted by Andrews et al. confirmed this method for a typical deep-freeze temperature of –20 °C (Andrews, 2004). However, the study was conducted with a lower initial bioburden ( $10^3$  CFU/g) and longer storage periods of 28 to 42 weeks (Andrews, 2004). Parker et al. showed that the reduction in contamination resulting from freezing at –20 °C could be accelerated further by vacuum packaging. After seven days, a log level reduction of three to four was observed for these samples. Concentrations continued to fall, and more rapidly than the conventionally packaged group, until the end of the study after 70 days (Parker et al., 1994).

Another effective method for reducing microbial load is the high-pressure processing of seafood. Experiments conducted with various vibrio species show that these are sensitive to high hydrostatic pressure applied at room temperature. In oysters, contamination with *V. vulnificus* was reduced by five log levels with the application of hydrostatic pressure at 250 MPa for two minutes (Koo et al., 2006). A figure of 300 MPa for three minutes was necessary to achieve the same results for oysters contaminated with *V. parahaemolyticus* (Cook, 2003). A more recent study conducted by Vu et al. in 2018 achieved similar results (Vu et al., 2018a). In this study, *V. vulnificus* proved to be the most sensitive species to the application of high levels of hydrostatic pressure for bioburden reduction (Vu et al., 2018a). To achieve a reduction in contamination by five log levels in each case, the following values and process times were required for the following *Vibrio* species at a temperature of 25 °C: i) *V. alginolyticus* and *V. cholerae*, 350 to 450 MPa for at least one minute; ii) *V. vulnificus*, 250 MPa for at least three minutes or 350 to 450 MPa for at least one minute; iii) *V. parahaemolyticus* 350 MPa for at least three minutes or 450 MPa for at least one minute (Vu et al., 2018a). Another physical method that is applied in particular in the case of bivalve shellfish is the process known as ‘depuration’. Depuration describes a procedure of rinsing with clean seawater

(which can be naturally sourced or artificially manufactured) under controlled conditions. The process is carried out immediately after the catch or harvest. The water used for depuration is either plain water at a defined temperature, water irradiated with ultraviolet light before application, or water treated with decontaminants such as chlorine, iodine or ozone (EO) (Lee et al., 2008). While this method has proven effective at removing human enterobacteria, findings are nonetheless inconsistent among studies examining reductions to vibrios autochthonously present in shellfish. Accordingly, it is not possible to make a reliable statement on the effective reduction of vibrios in these foods (Colwell and Liston, 1960; Vasconcelos and Lee, 1972; Eyles and Davey, 1984; Tamplin and Capers, 1992; Nordstrom et al., 2004; Ren and Su, 2006; Chae et al., 2009; Su et al., 2010).

Low-temperature pasteurisation is another method of reducing microbial load, which is utilised for seafood intended for raw consumption and oysters in particular. In this process, the shellfish are placed in plain seawater (natural or artificial) heated to 55 °C directly after the catch or harvest (and possibly depuration) for a period of five minutes (Andrews et al., 2000). As a result, temperatures of 48 to 50 °C are achieved within the bivalve shellfish (Andrews et al., 2000). In a study by Park and Chen, an initial microbial load of 10<sup>5</sup> CFU/g *V. parahaemolyticus* was successfully reduced to a value under the limit of detection (Andrews et al., 2000).

The US Food and Drug Administration (FDA) has also approved irradiation for the reduction of contamination in seafood (Food and Drug Administration, 2005a). Approved methods include irradiation with gamma rays and X-rays. A reduction in contamination with the microbe *V. parahaemolyticus* by six log levels was achieved at an irradiation intensity of 3.0 kilograys (kGy), both for the application of gamma rays and x-rays, in shrimps (Mahmoud, 2009a) and oysters (Jakabi et al., 2003). Similar results were achieved for the irradiation of oysters contaminated with *V. vulnificus* (Mahmoud, 2009b).

Maintaining an interior temperature of 70 °C in the food to be consumed for two minutes is another reliable method to ensure the inactivation of these pathogens.

6. Which standardised culture techniques and molecular protocols are suitable for the detection and analysis of the health risk posed by pathogenic *Vibrio* spp. (non-cholera vibrios) in food?

Detection methods for the three most significant human pathogenic vibrios (*V. parahaemolyticus*, *V. cholerae* and *V. vulnificus*) are provided both by the FDA (Kaysner et al., 2004) and the International Standards Organisation (ISO).

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 the detection of these three species. The method can be applied to products intended for human consumption and for use in animal feed. Environmental samples, taken in the context of food production or the handling of food products, can also be analysed with this method. 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 vibrios 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 the 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 population of vibrios and are frequently accompanied by a large number of other microorganisms from the Vibrionaceae family as well as 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 as vibrios. To suppress gram-positive bacteria and gram-negative Enterobacteriaceae (Kobayashi et al., 1963; Monsur, 1963), a thiosulfate-citrate-bile salts-sucrose (TCBS) agar is used, which simultaneously permits a differentiation between *V. cholerae*, *V. metschnikovii*, *V. fluvialis*, *V. furnissii* and *V. alginolyticus*, which can utilise sucrose, and the *Vibrio* species *V. mimicus*, *V. parahaemolyticus* and *V. vulnificus*, which cannot (Ceccarelli et al., 2019). A solid culture medium complementary to TCBS is used as the second solid medium. CHROMagar™ Vibrio (CVA) is frequently utilised here (Hara-Kudo et al., 2001), which contains a proprietary mixture of colourants. This serves as a substrate for the  $\beta$ -galactosidases from *V. parahaemolyticus* (colour: mauve), which is coloured to contrast strongly with *V. cholerae* and *V. vulnificus* (turquoise) and other *Vibrio* species (colourless to creamy, *V. alginolyticus*). This enables the species *V. parahaemolyticus* to be identified/distinguished, since this 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* (Høi et al., 1998).

As a final step, the presence of potential vibrios is confirmed using suitable biochemical and/or molecular methods (polymerase chain reaction (PCR), real-time PCR). Biochemical testing encompasses media that confirm the presence of vibrios on the one hand (arginine dihydrolase (ADH) broth, l-lysine decarboxylase (LDH) broth, indole), while also utilising combinations of specific media in order to determine the species (e.g.: ability to hydrolyse ONPG, reactions in sucrose, lactose, growth at various concentrations of salt). Commercially available tests are also suitable for the analysis of biochemical properties.

For the molecular detection of the most important human pathogenic vibrios, a variety of well-established PCR-based systems are available (Hill et al., 1991; Bej et al., 1999; Chun et al., 1999). The genes *vvh*, *toxR* or *tlh* and *sodB* are used to confirm *V. vulnificus*, *V. parahaemolyticus* and *V. cholerae*, respectively. While specific genes can also be queried using molecular methods to estimate the human pathogenic potential of the species *V. parahaemolyticus* (*tdh*, *trh*) and *V. cholerae* (*ctxA*, O1, O139), there is currently no definitive correlation between pathogenicity and the presence of a specific gene in the case of *V. vulnificus*, since this species has high genetic diversity.

In contrast to the ISO standard, detection methods for the three enteropathogenic vibrios in the FDA's Bacteriological Analytical Manual (BAM, chapter 9) are listed according to the species to be investigated (Kaysner et al., 2004). The scope of application is restricted here to foods and cosmetics. While the approach to the detection of *V. cholerae* and *V. vulnificus* is largely comparable with the methods described in the ISO standard, two additional detection options are described for *V. parahaemolyticus*. By utilising 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 of these methods are used routinely, since they are extremely time-consuming.

Despite options for rapid and reliable species identification, new technologies, such as matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI TOF MS), are not accounted for by ISO 21872 as a potential method for species confirmation

(Dieckmann et al., 2010). It can be assumed that the MALDI TOF MS technique will be included in future revisions of the standard (ISO 21872).

7. Which markers/indicators (e.g. genes or proteins) are suitable – e.g. as virulence factors for pathogenic strains – for facilitating the (rapid) detection of pathogenic vibrios within food production? With what level of confidence can these markers indicate a potential health risk associated with the consumption of food contaminated with vibrios (non-cholera vibrios)?

For the three enteropathogenic *Vibrio* species, markers or indicators for virulence are only available in the case of *V. parahaemolyticus* and *V. cholerae*.

*V. parahaemolyticus* strains that possess genes for the haemolysin TDH (thermostable direct haemolysin) and/or TRH (TDH-related haemolysin) are considered to be potentially enteropathogenic microbes (Nishibuchi and Kaper, 1995; Park et al., 2000). Food containing strains having these virulence markers must be subjected to a treatment process that inactivates these bacteria. A culture method is available for the direct detection of the TDH haemolysin on a special blood agar (Wagatsuma agar) by means of a lysis zone around the *V. parahaemolyticus* colonies (known as the Kanagawa phenomenon) (Miyamoto et al., 1969). However, this is not applicable for the detection of TRH haemolysins, since colonies with this haemolysin are negative on Wagatsuma agar (Honda et al., 1990). This culture method has since been replaced by PCR methods, which can reliably indicate presence or absence of the haemolysis genes *trh* or *tdh* in *V. parahaemolyticus* strains. Some of these PCR detection methods are also included in the ISO standard 21872-1.

In the case of *V. cholerae* detection in a food product, the aim is to clarify the presence of a potentially toxigenic strain from the O1 or O139 serogroups. While earlier methods utilised agglutination with commercial O1 and O139 sera, the detection of cholera toxin (typically the A subunit, i.e. the *ctxA* gene) is today achieved using PCR. Other supplementary PCR methods include the detection of genes from the biosynthesis of the O1 or O139 antigen, using a multiplex approach (Schirmeister et al., 2014). Negative PCR reactions for the cholera toxin gene indicate that these strains are not toxigenic. In the multiplex PCR approach, *V. cholerae* non-O1, non-O139 strains are reliably detected using a species-specific gene. On the other hand, there are no generally recognised virulence factors capable of identifying the enteropathogenic isolates from the non-O1, non-O139 group.

In the case of *V. vulnificus*, no unambiguous detection of virulence markers is possible. While the number of infections involving *V. vulnificus* remains low, an analysis of clinical strains has revealed the presence of wide-ranging genetic diversity between the strains (Jones and Oliver, 2009). Accordingly, the ISO standard 21872-1 only lists PCR detection methods capable of definitively identifying the species. PCR detection is achieved using primers that detect the *V. vulnificus* haemolysin (VVH). This gene is present in all isolates of the species.

#### Further information on the subject of vibrios from the BfR website

Consultant laboratory for vibrios: [https://www.bfr.bund.de/en/consultant\\_laboratory\\_for\\_vibrios-252894.html](https://www.bfr.bund.de/en/consultant_laboratory_for_vibrios-252894.html)



BfR 'Opinions app'

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The German Federal Institute for Risk Assessment (BfR) is a scientifically independent institution within the portfolio of the Federal Ministry of Food and Agriculture (BMEL) in Germany. The BfR advises the Federal Government and the States ('Laender') on questions of food, chemicals and product safety. The BfR conducts its own research on topics that are closely linked to its assessment tasks.

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