Potentially pathogenic *Vibrio* species in the German Bight, North Sea

by

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“Nothing in life is to be feared; it is only to be understood”

Marie Skłodowska-Curie

Table of Contents

Thesis summary

Potentially pathogenic *Vibrio* spp. are ubiquitous marine bacteria, autochthonous to estuaries and coastal waters. *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* are the main water-related pathogenic species, which are able to cause serious gastroenteritis, wound infections or septicaemia in human hosts. Infection cases caused by potentially pathogenic *Vibrio* spp. have become more frequent in northern temperate waters, mostly attributed to climate change related events. However, generally very little information is available on pathogenic *Vibrio* spp. in the North Sea and particularly in the German Bight.

Spatio-temporal occurrence, abundance and pathogenic characteristics of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* were investigated in a salinity gradient of the German Bight over the course of 14 months. Seasonal patterns of all three species with increased abundances during summer months were detected, while extended periods of warm seawater coincided with prolonged *Vibrio* spp. occurrences in the German Bight. Temperature and nitrite were found to be the significant factors explaining variations in *Vibrio* spp. abundances. This study revealed that environmental human pathogenic *Vibrio* spp. comprise multiple virulence-associated genes in the German Bight, especially in estuarine regions.

This study comprised the first investigation of the pathogen growth potentials of clinically relevant *V. vulnificus* and *V. cholerae* strains to determine the extent of *in vitro* growth in a broad range of physico-chemical conditions of surface waters originating from a salinity gradient in the German Bight. It has been demonstrated that clinically relevant *V. vulnificus* and *V. cholerae* strains, despite revealing different growth patterns, are capable of growth in most seawater samples under ambient physicochemical conditions. Growth kinetics, particularly that of *V. vulnificus,* showed strong temperature dependency when grown in seawater. While salinity had an influence on the population densities under high-nutrient conditions (broth), no salinity dependency was detected under seawater conditions.

Microplastic litter is a growing concern in aquatic ecosystems and serves as surfaces for microbial attachment. This study detected the presence of potentially pathogenic *Vibrio* spp., namely *V. parahaemolyticus*, on marine microplastics for the first time. Hence, there is

evidence that microplastic particles can serve as additional surfaces for attachment under non-optimal environmental conditions and that those might function as vectors for the enrichment and dispersal of pathogenic *Vibrio* spp..

The results of this thesis substantially increase the understanding of environmental drivers and the virulence diversity of potentially pathogenic *Vibrio* spp. in the German Bight. This thesis highlights the possibility of an emergence and establishment of new pathogenic strains in the German Bight and their possible dispersal over long distances on microplastic particles. Given the future predictions of climate variability, including further warming and longer warm seasons, heatwave events and heavy river discharge, a species-specific monitoring and risk assessment regarding potentially pathogenic *Vibrio* spp. in the German Bight is crucial.

General Introduction

The genus *Vibrio*

In 1854, John Snow studied the epidemiology of a cholera outbreak in England and assumed that the “cholera poison” propagated the outbreak, polluting a public well. Filippo Pacini described the first *Vibrio* species, *Vibrio cholerae,* while studying outbreaks of the cholera disease in Florence during the same period. Only thirty years later (1884), the first *V. cholerae* strain was isolated by Robert Koch (Thompson et al., 2004a).

The genus *Vibrio* belongs to the class of G*ammaproteobacteria* and consists of more than 100 species (Romalde et al., 2014). These bacteria are chemoorganotrophic, primarily facultative anaerobes, are gram-negative, appear as rod-shaped cells with a single polar flagellum (Farmer et al., 2005), and are ubiquitous to marine and estuarine environments. All *Vibrio* species possess two Chromosomes as a characteristic feature (Heidelberg et al., 2000). The large chromosome Chr1 carries all essential genes for growth and viability whereas the smaller chromosome (Chr2), coding for several metabolic and regulatory pathways, is considered more species-specific (Heidelberg et al., 2000). The two-chromosome genome structure is suggested to aid short generation times, a rapid replication and fast adaptation to changing environments, such as adapting to nutritional stresses by the increased expression of the small chromosome during intestinal growth (Xu et al., 2003, Yamaichi et al., 1999).

Potentially pathogenic *Vibrio* spp.

Some *Vibrio* species are common zoonotic agents towards molluscs (Paillard, 2004), corals (Vezzulli et al., 2010a) causing coral bleaching (Ben-Haim et al., 2003), and fish (Austin et al., 2005). Others are classified as human pathogens causing serious infections of different types (Morris Jr and Acheson, 2003). The genus *Vibrio* comprises at least 12 species known to cause human diseases, either intestinal like gastroenteritis or the Cholera, or extraintestinal such as septicaemia, wound infections or otitis. While many of those species are considered as weak pathogens causing rare infections (*V. alginolyticus*, *V. harveyi*, *V. mimicus*, *V. fluvialis*, and

more) (Thompson et al., 2004a), the most prominent human pathogenic species are *Vibrio parahaemolyticus, V. vulnificus* and *V. cholerae.*

V. cholerae is the etiological agent of the Cholera disease, representing a global burden between 1.4 and 4.3 million cases with about 21,000 – 143,000 deaths per year (WHO, 2017). The classical Cholera disease is caused by toxigenic *V. cholerae* strains capable of producing the Cholera toxin (CT) leading to severe watery diarrhea, electrolyte loss and fatal dehydration, when left untreated. More than 200 serotypes of *V. cholerae* are existing based on the serological variation of the somatic "O" antigen structure (Shimada et al., 1994). Hence, epidemic outbreaks of Cholera are only caused by two serotypes worldwide, O1 and O139. Infections with those epidemic strains are not common in the northern hemisphere and are usually travel-related (Schirmeister et al., 2014). However, non-O1/non-O139 *V. cholerae* are considered opportunistic pathogens and can cause sporadic cases of diarrhea, otitis and bacteraemia (Albuquerque et al., 2013, Deshayes et al., 2015, Huehn et al., 2014, Chowdhury et al., 2016). In rare cases, these infections are fatal in immunocompromised patients especially suffering from liver disease (Huhulescu et al., 2007, Patel et al., 2009) with a mortality of 33 % (Deshayes et al., 2015). Although specific virulence factors of non-O1/non-O139 *V. cholerae* strains enabling infections are barely elucidated, several accessory virulence-associated genes are commonly targeted for genetic characterization of clinical and environmental non-O1/non-O139 *V. cholerae* strains, including hemolysins, protein secretion systems and pathogenicity islands. Studies in different regions of the world reaching from South Africa (Abia et al., 2017) to Bangladesh (Hasan et al., 2013), the USA (Ceccarelli et al., 2015) and recently in Germany (Schwartz et al., 2019) detected accessory virulence-associated genes in environmental *V. cholerae*.

Vibrio vulnificus is often associated with fish and shellfish but poses also the most lethal foodborne pathogen with a fatality rate up to 50 %, the highest of any known foodborne pathogens. This organism can cause either seafood-related primary septicaemia when ingested or severe infections of open wounds. The disease fatality strongly correlates with patient age, gender and a decreased immune response, with an 80 % increased primary septicaemia risk for older aged males with chronic liver diseases (Baker-Austin and Oliver, 2018) especially due to elevated serum iron levels. Increasing incidents of *V. vulnificus* infections are occurring globally, especially in Europe, the USA and Asia (Hlady and Klontz,

1996, Baker-Austin et al., 2013). *V. vulnificus* strains can be classified into three biotypes based on their biochemical characteristics and phylogeny: The Biotype 1 constitutes predominantly clinical isolates, Biotype 2 is considered an eel pathogen but is also detected to cause human infections, whereas Biotype 3 strains are geographically limited to Israel. *V. vulnificus* strains are commonly differentiated in clinical (C) or environmental (E) types by targeting DNA sequence polymorphisms of the virulence-correlated gene (vcg) (Warner and Oliver, 1999) and 16S rRNA genes (type A and B) (Nilsson et al., 2003). While higher virulence was correlated with vcg C-type strains this attribute was not exclusive to those and cannot predict virulence (Thiaville et al., 2011), since up to 60 % of clinical isolates are found to belong to the E-type strains (16S rRNA type A, vcg E) in the Baltic Sea region (Bier et al., 2013). Thus, some virulence-associated genes encoding the N-acetylneuraminate lyase of the sialic acid catabolism cluster (*nanA*), the mannitol fermentation operon (*manIIA*) as well as the Pathogenicity Region XII (PRXII) were suggested for risk assessment purposes (Bier et al., 2015).

V. parahaemolyticus is the most common agents of seafood-borne illnesses, which are caused by the consumption of undercooked or raw shellfish in America and Asia (Su and Liu, 2007). The most distinctive virulence traits associated with gastroenteritis cases, the thermostable direct hemolysin (tdh) and the tdh-related hemolysin proteins are present in a small fraction of environmental *V. parahaemolyticus* strains (Honda and Iida, 1993, Nishibuchi et al., 1992). Epidemics of severe gastroenteritis were caused worldwide by *V. parahaemolyticus*. Especially the pandemic clonal strain O3:K6 emerged in 1996 and abruptly increased the number of gastroenteritis cases, first in south-east Asia and was later on detected worldwide (Nair et al., 2007, Matsumoto et al., 2000), even in temperate European waters (Baker-Austin et al., 2010; Martinez-Urtaza et al., 2005).

Ecology of human pathogenic *Vibrio* spp.

Vibrio spp. represent only ~1 % of the total microbial population in coastal waters (Thompson and Polz, 2006) but constitute up to 10 % of the readily culturable marine bacteria in these habitats (Eilers et al., 2000). Most species are halophilic, require 0.55 – 3.0 % NaCl for optimum growth (Farmer et al., 2005), participate in nitrogen fixation as well as chitin degradation and in the metabolism of algal polysaccharides (Thompson and Polz, 2006).

Vibrio spp. make use of survival strategies to withstand diverse physical, chemical, and biological stresses in the aquatic environment, which are especially prevalent in estuaries. That includes fluctuations in temperatures and nutrient, oxidative stress, bacteriophage predation, and protozoan grazing (Faruque and Nair, 2002, Matz et al., 2005). One of those strategies of potentially pathogenic *Vibrio* spp. is entering the viable-but-non-culturable (VBNC) state to endure low temperatures and starvation (Wong and Wang, 2004, Rao et al., 2014). In this dormant state, viability distinctly decreases, while morphological changes occur from rod to coccoid cell shapes (Fernández-Delgado et al., 2015) and gene regulation is significantly downregulated (Meng et al., 2015). *Vibrio* spp. can resuscitate when environmental conditions become favourable again (Fernández-Delgado et al., 2015), even after residing in the VBNC state for up to several years (Alam et al., 2007). Another survival strategy is switching between the free-living and the biofilm lifestyles by attachment to surfaces. Generally, *Vibrio* spp. tend to colonize marine surfaces like plants, a variety of organisms like zooplankton, phytoplankton, crustaceans, and algae or wood (Hood and Winter, 1997, Grimes et al., 2009b). Potentially pathogenic *Vibrio* spp. are mostly associated with chitinous plankton, which is considered an important reservoir of these bacteria in nature (Turner et al., 2009). Pathogenic *V. cholerae* strains, both O1 and non-O1 Serotypes, were found to be attached to the surfaces of copepods in natural waters (Huq et al., 1983). By attaching, *Vibrio* spp. can make use of nutrients released by the hosts or degrade the polymeric substances of the surfaces.

There is evidence for ecological specialization and long-term predictability of population-habitat associations in coastal *Vibrionaceae* (Szabo et al., 2013). However, fine-scale spatio-temporal dynamics in population structures are rather habitat-specific and influenced by sudden changes, such as shifts in eukaryotic plankton, climatic events or nutrient pulses. Accordingly, *Vibrio* spp. live in a “feast and famine” lifecycle, blooming when favourable conditions prevail leading to sudden increases in abundances (Chafee et al., 2018, Gilbert et al., 2012). Genotypic divergence in coastal potentially pathogenic *Vibrio* spp. are linked to phenotypic traits relevant to an elevated fitness in certain niche environments (Kirchberger et al., 2016, Keymer et al., 2007). Toxigenic *V. cholerae* O139 Serotype have been shown to maintain a relatively tight clonal structure. In contrast, non-toxigenic *V. cholerae* isolates exhibit a greater genetic diversity expanding in multiple clonal lineages in the environment (Zhang et al., 2014).

Surface-associated potentially pathogenic *Vibrio* spp. abundances can be higher than free-living abundances in coastal areas (Oberbeckmann et al., 2018). Exemplarily, shellfish can contain relatively high levels of *V. parahaemolyticus*, including pathogenic strains possessing the virulence markers tdh and trh (Parveen et al., 2008, Martinez-Urtaza et al., 2008). In contrast, potentially pathogenic *Vibrio* spp. are also found to readily adopt the free-living lifestyle when favourable conditions prevail (Eiler et al., 2006). The established literature provides a broad background for understanding the potential environmental factors influencing *Vibrio* spp. abundances in aquatic environments, but it remains difficult to identify consistencies across studies. Various biotic and abiotic factors are reported to trigger potentially pathogenic *Vibrio* spp. occurrences and abundances in coastal and estuarine environments, as reviewed by Takemura et al. (2014), (Johnson, 2015). Countless studies investigated the complex ecology of environmental potentially pathogenic *Vibrio* spp., targeting different spatio-temporal extents and resolutions. Those comprise genus-specific environmental dependency investigations (Vezzulli et al., 2016), which can mask important species-specific dynamics, with an increasing trend towards finer resolutions with species-specific approaches (Eiler et al., 2007). Studies were conducted in different climatic regions (tropical and temperate), covering different lifestyles (attached or free-living) (Huq et al., 2005, Pfeffer et al., 2003, Oberbeckmann et al., 2011b). Targeting concomitant environmental effects on potentially pathogenic *Vibrio* spp. abundances, studies use diverse statistical approaches with different complexities, ranging from tests for significance (Liang et al., 2019), multiple linear regressions (Oberbeckmann et al., 2012), additive models (Vezzulli et al., 2016) to multivariate analysis (Jesser and Noble, 2018). Prominent environmental parameters influencing *Vibrio* spp. communities and abundances are temperature, salinity, plankton abundance or nutrients (Blackwell and Oliver, 2008; Caburlotto et al., 2010; Drake et al., 2007; Martinez-Urtaza et al., 2008; Thompson et al., 2004b; Turner et al., 2009; Vezzulli et al., 2009, Oberbeckmann, 2011b). However, the only consistent environmental association shared by the majority of studies is seawater temperature as a key factor influencing the presence and abundance of *Vibrio* spp. (Vezzulli et al. 2010, Schets et al. 2010, Baker-Austin et al. 2013).

In northern European seawaters specifically, *V. parahaemolyticus, V. vulnificus* and *V. cholerae* occurrences were addressed in several studies in (Bauer et al., 2006, Böer et al., 2013, Collin and Rehnstam-Holm, 2011, Ellingsen et al., 2008, Lhafi and Kuhne, 2007, Oberbeckmann et al., 2011b, Schets et al., 2011, Schets et al., 2010). However, only in few spatially or temporally

limited studies investigated environmental drivers of potentially pathogenic *Vibrio* spp. in the German Bight (Oberbeckmann et al. 2011b, Böer et al. 2013).

There is a considerable public health and economic interest in predicting increased abundances of potentially pathogenic *Vibrio* spp. (Stewart et al., 2008). To monitor and forecast conditions suitable for increased *Vibrio* abundances, the European Centre for Disease Prevention and Control (ECDC) developed a web-based platform, the *Vibrio* Map Viewer. This quasi–real-time platform is using remote sensing data, such as surface temperature and salinity. However, this forecast platform is based on the Baltic Sea, which might not apply to other environmental conditions. The US Centre for disease control and prevention (CDC) initiated the Cholera and Other Vibrio Illness Surveillance (COVIS) system, for reporting human infections associated with pathogenic *Vibrio* spp. To date, there is no surveillance system operating for European countries, while only toxigenic travel-related *V. cholerae* infections are among the notifiable illnesses at local health authorities.

The German Bight: A Wadden sea in the North Sea

The North Sea is a semi-enclosed continental shelf sea stretching between the northern European mainland and the Atlantic Ocean. In response to tides, westerly winds and freshwater or oceanic (Atlantic) water inflow, the North Sea is subjected to an anti-clockwise long-term water circulation along the coast (Sündermann and Pohlmann, 2011). The strength of westerly winds, precipitation and the intensity of seasons in the North Sea region are linked to the Northern Atlantic Oscillation (NOA) (Emeis et al., 2015), a weather phenomenon caused by atmospheric pressure differences between the Icelandic low and the Azores high. The North Sea is located in the temperate latitudes (51 °N to 62 °N) and is exposed to pronounced seasonal changes with average temperatures ranging from 6°C to 17°C. With a depth ranging from < 40 m in the shallow southern region (Dogger Bank), the North Sea reaches up to 200 m depth at the shelf edge (Howarth, 2001). An extended gradient stretches from marine conditions in the northern areas towards the brackish conditions in the coastal zones (Emeis et al., 2015). In offshore regions salinity varies between 32 – 35, while salinities around 15 – 25 prevail in estuarine regions. Parts of the northern North Sea is seasonally stratified due to heat input whereas the water column in the southern North Sea, particularly the German

Bight, is a well-mixed system throughout the year due to shallow depths and strong tides (Howarth, 2001). The extent and characteristic of the mixing zones are defined by the oceanic water inflow and irregular river input of up to 300 km^2 per year (Emeis et al., 2015).

The German Bight is located in the south-eastern North Sea and comprises the world's largest tidal flat area, the Wadden Sea. Enclosed by the industrial countries Denmark, Germany and the Netherlands, the German Bight is highly influenced by freshwater discharge from the rivers Elbe and Weser, constantly being supplied with nutrients and raising its productivity up to 430 g C m^{-2} a^{-1} (Rick et al., 2006). The mixing of fresh- and marine waters in estuaries typically leads to high spatial variability with respect to physico-chemical environmental factors, especially for temperature, salinity, pH and organic substances, leading to strong geographical gradients (Atlas and Bartha 1987). The German Bight is exposed to pronounced anthropogenic influences ranging from extensive shipping traffic (van der Meer et al., 2016), pollution (Schwarzbauer et al., 2000), tourism (Kelletat, 1993) and recreational activities. Climate change has a big impact on the North Sea, one of the most rapidly warming seas in the world (Belkin, 2009), with a trend of 0.4 °C per decade (Emeis et al., 2015). For the German Bight alone, a warming trend in annual mean temperature of at least 1.67 °C has been detected since 1962 (Wiltshire et al., 2010). Furthermore, extended warm seawater periods and more frequent extreme climatic events, such as heatwaves and heavy precipitation events are expected in the North Sea in the future (Christidis et al., 2015, Brennholt et al., 2014).

Study Aims

Chapter I:

Potentially human pathogenic *Vibrio* spp. in a coastal transect: Occurrence and multiple virulence factors

1. What are the spatio-temporal occurrence and abundance patterns of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* in a salinity gradient of the German Bight over the course of 14 months?
2. Which physico-chemical factors are the drivers of elevated potentially human pathogenic *Vibrio* spp. abundances in this coastal region?
3. Which toxin and virulence-associated genes of these species are present and how are they distributed in a spatio-temporal dimension?

Chapter II:

***In vitro* growth potentials of clinically relevant *V. cholerae* and *V. vulnificus* strains in a salinity gradient in the German Bight (North Sea)**

1. To which extent does seawater originating from the German Bight support the *in vitro* growth of clinically relevant *V. cholerae* and *V. vulnificus* strains?
2. Which physico-chemical factors have an impact on the *in vitro* growth kinetics and population densities of those strains?
3. How does nutrient limitation influence the growth behaviour of clinically relevant *Vibrio* spp. strains towards salinity and temperature fluctuations?

Chapter III:

Dangerous hitchhikers? Evidence for potentially pathogenic *Vibrio* spp. on microplastic particles

1. Are potentially pathogenic *Vibrio* spp. present on marine microplastic particles floating in the North and Baltic Sea?
2. If they are present, do they comprise virulence genes?
3. Are these species also present in the surrounding seawater?

Study Outline

The present thesis consists of an introductory overview, three research articles (Chapters) based on the addressed study aims, and a final general discussion. A short overview of the Chapters follows.

Chapter I

Potentially human pathogenic *Vibrio* spp. in a coastal transect: Occurrence and multiple virulence factors

Authors: Sidika Hackbusch, Antje Wichels, Luis Gimenez, Hilke Döpke, Gunnar Gerdts

Published in: *Science of the total Environment*. 10 March 2020; https://doi.org/10.1016/j.scitotenv.2019.136113

This manuscript describes the occurrence, abundance and virulence characteristics of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* in a salinity gradient of the German Bight. The influence of environmental parameters on the abundance and pathogenic occurrence of potentially pathogenic *Vibrio* spp. is also presented. Sidika Hackbusch collected and genetically characterized all samples, supported by Hilke Döpke. Dr. Luis Gimenez conducted generalized additive mixed models. Data evaluation and manuscript writing were carried out by Sidika Hackbusch under the guidance of Dr. Antje Wichels and Dr. Gunnar Gerdts.

Chapter II

In vitro growth potentials of clinically relevant _V. cholerae_ and _V. vulnificus_ strains in a salinity gradient in the German Bight (North Sea)

Authors: Sidika Hackbusch, Antje Wichels, Gunnar Gerdts

To be published in: mBio

This manuscript presents the pathogen growth potentials of clinically relevant *V. cholerae* and *V. vulnificus* strains *in vitro* in surface waters of the German Bight. The influence of environmental parameters on growth factors is evaluated. Sidika Hackbusch collected all samples and conducted growth assays. Data evaluation, statistical analysis and manuscript writing were carried out by Sidika Hackbusch under the guidance of Dr. Antje Wichels and Dr. Gunnar Gerdts.

Chapter III

Dangerous hitchhikers? Evidence for potentially pathogenic _Vibrio_ spp. on microplastic particles

Authors: Inga V. Kirstein*, Sidika Kirmizi*, Antje Wichels, Alexa Garin-Fernandez, Rene Erler, Martin Löder, Gunnar Gerdts

Published in: *Marine Environmental Research*. September 2016;
https://doi.org/10.1016/j.marenvres.2016.07.004

This manuscript investigates the occurrence of potentially pathogenic *Vibrio* spp. on floating microplastics. It is shown that the potentially pathogenic *V. parahaemolyticus* were detected on a number of microplastic particles from North and Baltic Sea. This study integrates the sample analyses from two cruises, HE409 and HE430. Sidika Kirmizi (HE409) and Inga V. Kirstein (HE430) collected and analysed the microplastic and surface water samples. Data evaluation and manuscript writing were carried out by Inga V. Kirstein and Sidika Kirmizi under the guidance of Dr. Antje Wichels and Dr. Gunnar Gerdts. Alexa Garin-Fernandez (HE430) and Dr. Rene Erler (HE430) assisted during MALDI TOF analysis. Microplastic identification by ATR FTIR was carried out under the guidance of Dr. Martin Löder.

Chapter I

Potentially human pathogenic *Vibrio* spp. in a coastal transect: Occurrence and multiple virulence factors

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Keywords: *V. parahaemolyticus*, *V. vulnificus*, Non-O1/non-O139 *V. cholerae*, Generalized additive mixed model, North Sea

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Abstract

An increase in human *Vibrio* spp. infections has been linked to climate change related events, in particular to seawater warming and heatwaves. However, there is a distinct lack of research of pathogenic *Vibrio* spp. occurrences in the temperate North Sea, one of the fastest warming seas globally. Particularly in the German Bight, *Vibrio* investigations are still scarce. This study focuses on the spatio-temporal quantification and pathogenic characterization of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* over the course of 14 months. Species-specific MPN-PCR (Most probable number – polymerase chain reaction) conducted on selectively enriched surface water samples revealed seasonal patterns of all three species with increased abundances during summer months. The extended period of warm seawater coincided with prolonged *Vibrio* spp. occurrences in the German Bight. Temperature and nitrite were the factors explaining variations in *Vibrio* spp. abundances after generalized additive mixed models. The specific detection of pathogenic markers via PCR revealed *trh*-positive *V. parahaemolyticus*, pathogenic *V. vulnificus* (*nanA, manIIA, PRXII*) and *V. cholerae* serotype O139 presence. Additionally, spatio-temporally varying virulence profiles of *V. cholerae* with multiple accessory virulence-associated genes, such as the El Tor variant hemolysin (*hlyAET)*, acyltransferase of the repeats-in-toxin cluster (*rtxC)*, Vibrio 7th pandemic island II (*VSP-II)*, Type III Secretion System (TTSS) and the Cholix Toxin (*chxA)* were detected. Overall, this study highlights that environmental human pathogenic *Vibrio* spp. comprise a reservoir of virulence-associated genes in the German Bight, especially in estuarine regions. Due to their known vast genetic plasticity, we point to the possible emergence of highly pathogenic *V. cholerae* strains. Particularly, the presence of *V. cholerae* serotype O139 is unusual and needs urgent continuous surveillance. Given the predictions of further warming and more frequent heatwave events, human pathogenic *Vibrio spp.* should be seriously considered as a developing risk to human health in the German Bight.

Introduction

Vibrio parahaemolyticus, V. vulnificus and *V. cholerae* are natural inhabitants of warm coastal and estuarine waters. Yet, single strains can be pathogenic to humans when displaying certain virulence traits. Typically, *Vibrio* outbreaks occur in tropical or subtropical regions or coincide with seawater warming conditions on a global scale. Human pathogenic *Vibrio* are becoming increasingly significant in temperate waters of northern latitudes, including in northern European regions like the Baltic Sea (Baker-Austin et al., 2013, Semenza et al., 2017). The most distinctive characteristics of *V. parahaemolyticus* strains causing infections are the thermostable hemolysin (*tdh*) and *tdh*-related hemolysin (*trh*) genes (Honda and Iida, 1993, Nishibuchi et al., 1992). Pathogenic *V. parahaemolyticus* is the leading cause for gastroenteritis in America and Asia related to seafood consumption (Su and Liu, 2007). Of special concern is the recent increase in these infection cases in temperate European waters (Baker-Austin et al., 2010, Roux et al., 2015, Ceccarelli et al., 2013). *Vibrio vulnificus* is the most lethal foodborne pathogen with a fatality rate up to 50 %; it causes seafood-related primary septicaemia and severe secondary wound infections. This pathogen is gaining international significance and is emerging in temperate northern European waters (Baker-Austin et al., 2013). While no single pathogenic gene is defined to be the causative agent for *V. vulnificus* infections, the virulence-associated genes encoding the N-acetylneuraminate lyase of the sialic acid catabolism cluster (*nanA*), the mannitol fermentation operon (*manIIA*), as well as the Pathogenicity Region XII (PRXII), were suggested for risk assessment purposes (Bier et al., 2015). *Vibrio cholerae* is the causative agent for Cholera epidemics worldwide. However, among more than 200 serotypes, only the *V. cholerae* serotypes O1 and O139 are responsible for those epidemics. Toxigenic *V. cholerae* infections with strains capable of producing the Cholera Toxin (CT) and the toxin co-regulated pilus (TCP), are not common in northern Europe and are usually travel-related. However, sporadic cases of diarrhea, otitis and bacteraemia can be caused by non-O1/non-O139 *V. cholerae* (Albuquerque et al., 2013, Deshayes et al., 2015, Huehn et al., 2014). Recently, a urinary tract infection was reported in south Australia which was caused by non-toxigenic non-O1/non-O139 *V. cholerae* (Leong et al., 2018), expanding its "infection routes" besides the usual intestinal, wound and ear infections. Although specific virulence factors of non-O1/non-O139 *V. cholerae* strains enabling infections are barely elucidated, several accessory virulence-associated genes are commonly targeted for genetic

characterization of clinical and environmental non-O1/non-O139 *V. cholerae* strains, including toxins, hemolysins, protein secretion systems and pathogenicity islands.

The German Bight is located in the south-eastern region of the North Sea, a temperate, semi-enclosed European continental shelf sea. Being a hydrodynamically and ecologically complex European sea, the North Sea is subjected to various direct anthropogenic influences such as tourism (Kelletat, 1993), shipping traffic (van der Meer et al., 2016) and pollution (Schwarzbauer et al., 2000). Currently, the North Sea is one of the most rapidly warming seas in the world (Belkin, 2009) with a trend of 0.4 °C per decade (Emeis et al., 2015). Several studies reported the presence of environmental potentially pathogenic *Vibrio* spp. in offshore surface waters of this region (Oberbeckmann et al., 2011b), as well as on recreational beaches (Böer et al., 2013), in sediments, oysters and hitchhiking on microplastic particles (Kirstein et al., 2016). As an attempt to estimate and prevent future outbreaks, numerous investigations of environmental dependencies of *Vibrio* spp. abundances covering different ecosystems and temporal resolutions worldwide show a great variety of significant environmental predictors (Takemura et al., 2014, Johnson, 2015). In the German Bight, studies of environmental predictors of *Vibrio* have been focusing on coastal recreational beaches (Böer et al., 2013) or on a single offshore site (Oberbeckmann et al., 2012, Oberbeckmann et al., 2011b). A comprehensive spatio-temporal study of the three potentially pathogenic *Vibrio* spp. is currently missing in this region. So far, environmental *V. cholerae* characterization in the German Bight was only covered by one recent study based on isolation from 2009 to 2014 (Schwartz et al., 2019). The authors detected a huge variety of accessory virulence-associated genes in *V. cholerae* strains from German recreational beaches in the North Sea (Schwartz et al., 2019), however, the spatio-temporal distributions and environmental dependencies of these potentially pathogenic *Vibrio* spp. are widely unknown in this region. To this end, spatio-temporal detection (qualitatively and quantitatively) and pathogenic characterization of the three species *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* was conducted in a coastal region with high anthropogenic pressure, along a transect from the Elbe Estuary into the German Bight, terminating close to the island of Helgoland. The focus of this paper is on the environmental conditions associated with elevated human pathogenic *Vibrio* spp. abundances and the spatio-temporal occurrence of virulence-associated genes of these species.

Materials and Methods

Sample collection and environmental parameters

Surface water samples were collected on monthly research cruises (May to October 2015 and May 2016 to January 2017) along a 62 km transect through the German Bight, in the south-eastern North Sea. This transect extends from the Elbe estuary to Helgoland (Figure 1, Table A.1) and covers six sampling sites (I – VI).

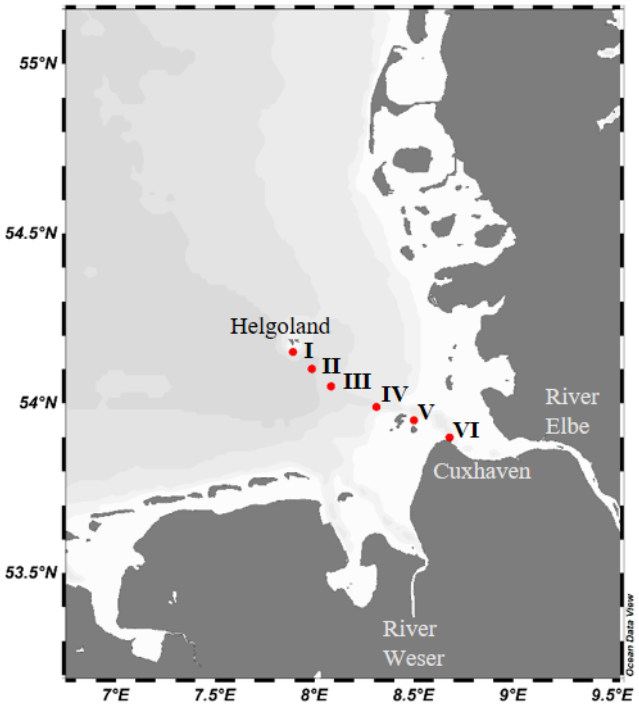

Figure 1: Sampling sites in the Elbe transect reaching from the island Helgoland [I] to the Elbe estuary [VI] of the south-eastern North Sea (German Bight) (Schlitzer, R., Ocean Data View, https://odv.awi.de, 2018)

At each site, surface water samples were taken with a rosette water sampler equipped with a CTD Probe (CTD90, Sea and Sun Technology, Germany) at a depth of approximately 1 meter. Water temperature, salinity, chlorophyll α (chl α), coloured dissolved organic matter (cDOM), dissolved oxygen (DO) and turbidity were recorded simultaneously. Additional environmental data including nitrite (NO_2), nitrate (NO_3), ammonium (NH_4), silicate (SiO_2) and phosphate (PO_4) were obtained in parallel as part of the Helgoland Roads time series (Wiltshire et al., 2008).

Sample preparation and enrichment of *Vibrio* spp.

MPN-PCR (Most probable number - polymerase chain reaction), commonly used for *Vibrio* spp. quantification in seafood samples (Luan et al., 2008), was applied in this study following the approach of Cantet et al. (2013) for surface water samples. For the 3-tube MPN-PCR quantification approach, surface water was concentrated onto 0.45 µm sterile membrane filters (ACN, Sartorius Stedim biotech, US) in different volumes (0.1 ml, 1 ml, 10 ml, 100 ml an 1 l) in three replicates each. After filtration, membrane filters were immediately transferred into tubes containing 9 ml sterile alkaline peptone water (APW) and incubated on board at 37 °C for 48 h. Subsequently, 1 ml of each APW enrichment culture was centrifuged at 12.000 rpm for 5 min and the pellet suspended in 380 µl STE buffer for later DNA extraction.

MPN-PCR quantification of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* and detection of virulence-associated genes

DNA was extracted using a chemical lysis and extraction protocol, followed by isopropanol precipitation according to Sapp et al. (2007). Prior to PCR reactions, DNA quantity and quality was determined photometrically (TECAN infinite M200, Switzerland). *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* were identified by species-specific multiplex PCRs targeting the highly conserved regulatory genes *Vp toxR* (*V. parahaemolyticus*), *vvhA* (*V. vulnificus*) and *Vc toxR* (*V. cholerae*) according to Neogi et al. (2010). PCR reactions were performed sequentially starting from the three DNA extracts representing the largest sample volume (1000 ml) until no PCR product was detected in any replicate of the respective MPN volume. Positive PCR results were recorded and the abundance of each species (MPN * L^{-1}) was calculated by using the MPN table of Sutton (2010).

Positive samples for each species were subsequently analysed for the presence of further virulence-associated genes. PCRs were conducted in at least duplicates and primers and PCR conditions were adapted from the respective literature (Table 1). For all PCRs 20 ng of template DNA was used in a 20 – 25 µl PCR mixture.

Table 1: List of applied primer sets. Target species and genes, primer sequences, amplicon sizes, primer concentrations, annealing temperatures and references.

Target species	Target gene	Primer	Sequence 5' - 3'	Amplicon Size [bp]	Conc. [µmol/l]	Annealing [°C]	References
Vc	*Vc toxR*	Vc toxR 403F	GAAGCTGCTCATGACATC	275	0.5	55	(Neogi et al., 2010)
		Vc toxR 678R	AAGATCAGGGTGGTTATTC				
Vv	*vvhA*	VvhA 870F	ACTCAACTATCGTGCACG	366	0.3	55	(Neogi et al., 2010)
		VvhA 1236R	ACACTGTTCGACTGTGAG				
Vp	*Vp toxR*	Vp toxR 325F	TGTACTGTTGAACGCCTAA	503	0.15	55	(Neogi et al., 2010)
		Vp toxR 828R	CACGTTCTCATACGAGTG				
Vv	*nanA*	VVA1199F	TKATCGCCGCTCCYCATACA	745	0.25	58	(Bier et al., 2013),
		VVA1199R	GCAACGCCACCGTATTCAAC				(Lubin et al., 2012)
Vv	*manIIA*	Man IIA F	GATGTTGGTGAACAACTTCTCTGC	234	0.25	61	(Froelich and Oliver, 2011)
		Man IIA R	TCTGAAGCCTGTTGGATGCC				
Vv	*PRXIIpres*	VVA1636F	TGTCCACGACTTGAACACG	1547	0.25	56	(Bier et al., 2013),
	(VVA1636/37)	VVA1637R	AACATCAACCAGCGAGTCGAA				(Cohen et al., 2007)
Vv	*PRXIIabs*	VVA1612bF	TGTGGAGAGCGGCAAGATCAA	1200	0.25	65	(Bier et al., 2013),
	(VVA1612/37)	VVA1637R	AACATCAACCAGCGAGTCGAA				(Cohen et al., 2007)
Vp	*tdh*	tdhD3F	CCACTACCACTCTCATATGC	425	0.375	62	(Tada et al., 1992)
		tdhD1R	CCATCTGTCCCTTTTCCTGC				
Vp	*trh*	trhF R2	GGCTCAAAATGGTTAAGCG	250	0.375	62	(Tada et al., 1992)
		trhR R6	CATTTCCGCTCTCATATGC				
Vc	*O139*	O139rfb-F	AGCCTCTTTATTACGGGTGG	449	0.16	59	(Mantri et al., 2006),
		O139rfb-R	GTCAAACCCGATCGTAAAGG				(Albert et al., 1997)
Vc	*O1*	O1rfb-F	GTTTCACTGAACAGATGGG	192	0.625	59	(Mantri et al., 2006),
		O1rfb-R	GGTCATCTGTAAGTACAAC				(Hoshino et al., 1998)
Vc	*ctxA*	ctxA1	CTCAGACGGGATTTGTTAGGCACG	301	0.16	59	(Nandi et al., 2000),
		ctxA2	TCTATCTCTGTAGCCCCTATTACG				(Chatterjee et al., 2009)
Vc	*hlyAET*	hlyA-744F	GAGCCGGCATTCATCTGAAT	481	0,2	60	(Rivera et al., 2001)
		hlyA-1184R	CTCAGCGGGCTAATACGGTTTA				

Vc	*rtxC*	VC1450-F	TGCAAATCTCACATTAGCGCA	430	0,2	55	(Schirmeister et al., 2013),
		VC1450-R	CCACTGCACCTTTCGGATACA				(Chow et al., 2001)
Vc	*chxA*	VC-chxA-F	TGTGTGATGATGCTTCTGG	2000	0,2	52	(Awasthi et al., 2013)
		VC-chxA-R	TTATTTCAGTTCATCTTTTCGC				
Vc	*VSP-II*	Vch-VspII-fo	TGCCCATTCCGCTAAGTGTTC	800	0,2	54	(Rahman et al., 2008),
		Vch-VspII-re	GCAAAAGCACTGCGTAAACTG				(Dziejman et al., 2002)
Vc	*tcpAET*	tcpA-F	CACGATAAGAAAACCGGTCAAGA	451	0,2	60	(Rivera et al., 2001)
		Class-ET (72F)	G				
		tcpA-R	CGAAAGCACCTTCTTTCACGTTG				
		ET (477R)					
Vc	*vcsC2*	TTSS_vcsC2-A	CGTTACCGATGCTATGGGT	535	0,2	60	(Chatterjee et al., 2009)
		TTSS_vcsC2-B	AGAAGTCGGTTGTTTCGGTAA				
Vc	*vcsN2*	TTSS_vcsN2-A	CAGTTGAGCCAATTCCATT	484	0,2	55	(Chatterjee et al., 2009)
		TTSS_vcsN2-B	GACCAAACGAGATAATG				
Vc	*vspD*	TTSS_vspD-A	AACTCGAAGAGCAGAAAAAAGC	422	0,2	55	(Chatterjee et al., 2009)
		TTSS_vspD-B	CTTCCCGCTTTTGATGAAATG				
Vc	*vcsV2*	TTSS_vcsV2-A	TTTGGCTCACTTGATGGG	742	0,2	55	(Chatterjee et al., 2009)
		TTSS_vcsV2-B	GCCACATCATTGCTTGCT				

Vibrio cholerae (Vc), Vibrio vulnificus (Vv), Vibrio parahaemolyticus (Vp)

Positive and negative control strains used are listed in table 2. All *V. parahaemolyticus* positive samples (*Vp toxR*$^{+}$) were tested for the presence of *tdh* (thermostable hemolysin) and *trh* (*tdh*-related hemolysin) according to Tada et al. (1992). *V. vulnificus* positive samples (*VvhA*$^{+}$) were tested for *nanA* (N-acetylneuraminate lyase of sialic acid catabolism cluster) (Bier et al., 2013, Lubin et al., 2012*)*, *manIIA* (mannitol fermentation operon) (Froelich and Oliver, 2011) and PRXII (Pathogenicity Region XII) (Bier et al., 2013, Cohen et al., 2007). Two PCRs were conducted to detect the PRXII region by targeting the gene *VVA1636/37,* the 3' chromosomal insertion site and partial chondroitinase AC lyase gene (PRXII^{+}). Samples positive for this gene are considered to carry the Pathogenicity Region XII. Additionally, the *VVA1612/37* flanking region gene was targeted to confirm the absence of the entire genomic region (PRXIIabs) (Cohen et al., 2007). *V. cholerae* positive samples (*Vc toxR*$^{+}$) were genetically characterized by a multiplex PCR detecting the serotype specific *rfb* O1 and O139 and the Cholera Toxin gene *ctxA* (Mantri et al., 2006, Nandi et al., 2000, Chatterjee et al., 2009). For a more detailed characterization of *V. cholerae*, following virulence-associated genes were targeted: *tcpAET* (El Tor allele toxin co-regulated pilus) and *hlyAET* (El Tor hemolysin) (Rivera et al., 2001), *rtxC* (acyltransferase of the repeats-in-toxin cluster) (Schirmeister et al., 2014, Chow et al., 2001), *chxA* (Cholix Toxin) (Awasthi et al., 2013), *VSP-II* (Vibrio 7th pandemic island II) (Rahman et al., 2008, Dziejman et al., 2002*)* and TTSS (Type III Secretion System) targeting the genes *vcsC2*, *vcsN2*, *vspD* and *vcsV2* (Chatterjee et al., 2009). PCR product sizes were confirmed with a MultiNA Microchip electrophoresis system (MCE-202 MultiNA, Shimadzu Biotech) in at least duplicates. Sanger sequencing performed at QIAGEN additionally confirmed O139 and PRXIIpres (*VVA1636/37*) PCR amplicons. Quality checks and assembly of reads were done with the software AlignIR comparing DNA sequences against the GenBank nucleotide database.

Table 2: Positive and Negative control strains for conducted PCR reactions

Target species	Target gene	Positive control strains	Negative control strains
Vc	*Vc toxR*	CH 111/ CH 187 / CH 258	ATCC 25919 (Vh)
Vv	*vvhA*	ATCC 27562	ATCC 25919 (Vh)
Vp	*Vp toxR*	RIMD 2210633	ATCC 25919 (Vh)
Vv	*nanA*	VN-94 / VN-207 / VN-108	ATCC 25919 (Vh)
	manIIA	VN-94	VN-108 (Vv) / ATCC 25919 (Vh)
	PRXIIpres (*VVA1636/37*)	VN-94 / VN-102 / VN-130	ATCC 27562 (Vv) / ATCC 25919 (Vh)
	PRXIIabs (*VVA1612/37*)	VN143	VN-94 (Vv) / ATCC 25919 (Vh)
Vp	*tdh*	RIMD 2210633 / CM12	ATCC 25919 (Vh)
	trh	CM24 / CM12	ATCC 25919 (Vh)
Vc	*O139*	CH 187	ATCC 25919 (Vh)
	O1	CH 111 / CH 258	ATCC 25919 (Vh)
	ctxA	CH 187 / CH 258	ATCC 25919 (Vh)
	hlyAET	MO45 / VN-0300	ATCC 25919 (Vh)
	rtxC	MO45 / VN-0300	ATCC 25919 (Vh)
	chxA	VN-0300	MO45 (Vc) / ATCC 25919 (Vh)
	VSP-II	MO45	VN-0300 (Vc) / ATCC 25919 (Vh)
	tcpAET	MO45	VN-0300 (Vc) / ATCC 25919 (Vh)
	TTSS (*vcsC2, vcsN2, vspD, vcsV2*)	VN-0300	MO45 (VC) / ATCC 25919 (Vh)

***Vibrio cholerae* (Vc), *Vibrio vulnificus* (Vv), *Vibrio parahaemolyticus* (Vp), *Vibrio harveyi* (Vh)**

Data Analysis and Statistics

Pairwise correlations between *Vibrio* spp. abundances and environmental parameters were calculated by Spearman rank order correlation at a significance level of $p < 0.05$. Differences of environmental parameters among the categories sampling site (I – VI) and month (January – December) were tested for significance by Kruskal-Wallis one-way analysis of variance on ranks followed by the Dunn's *post hoc* pairwise multiple comparison between categories (SigmaPlot 12) to reveal spatial and temporal trends of environmental parameters. Environmental dependence of each virulence-associated gene occurrence was tested with the non-parametric rank sum test Mann Whitney U test on binary data (presence/absence) (STATISTICA StatSoft Version 9.1). Plots were generated using the software R and SigmaPlot 12. Contour plots were constructed using the software Surfer 12 with kriging as spatial interpolation.

Spatial and temporal fluctuations in *Vibrio spp.* abundances were modelled from concomitant variation in environmental parameters, using general additive mixed models (GAMM: Wood (2006). The analysis was carried out using the R package *mgcv* (Wood, 2011). The fixed component consisted of environmental parameters included in an additive manner as a smooth based on the thin plate spline. Model selection was carried out only on the fixed component, using maximum likelihood (ML) fitting by evaluating the F-test of the summary component of the gam object. Estimated degrees of freedom were checked to identify linear relationships between predictor and response variables; in the case of which, the models were refitted with such variables as linear predictors. The full model had 'month of sampling' as a random intercept and Gaussian residuals (checked through qq-plots). Model outputs were visualized using the package mgcViz (Fasiolo et al., 2018). Data points in which concurrent environmental data were not available were excluded from all analysis as well as for model development.

Results

Environmental conditions

Spatial and temporal trends of environmental parameters were detected in this study (Figure A.1). Significant spatial differences among sampling sites were identified for salinity (H = 58), Chl α (H = 16.7), turbidity (H = 61.9), cDOM (H = 59.8), SiO_4 (H = 29.9), PO_4 (H = 31.5), NO_2 (H = 33.0) and NO_3 (H = 42.8) by Kruskal Wallis one-way ANOVA tests (Table A.2). Salinity decreased significantly from offshore (I – IV) to coastal sites (V – VI) (Figure A.1) and had a sudden drop in August 2015 with peaks in June and October 2015 and December 2016 (Figure A.1). Salinity showed negative collinearity with turbidity, cDOM, SiO_4, PO_4, NO_2, NO_3 and NH_4 with strong correlations especially with cDOM (r = - 0.99), turbidity (r = - 0.75) and NO_3^- (r = - 0.74) (Table A.3). Positive collinearity was detected between the parameters chl α, turbidity, cDOM and all inorganic nutrients, with strong correlations especially between SiO_4 and PO_4 (r = 0.80), turbidity and cDOM and NO_3 (r = 0.80, r = 0.76), NO_2 and NO_3 (r = 0.71), NO_2 and SiO_4 (r = 0.66) and between NO_2 and PO_4 (r = 0.68).

Significant temporal differences among sampling months were identified for temperature (H = 66.1), DO (H = 57.8) and for NH_4 (H = 35.5). Additional to their spatial variations, Chl α (H = 30.5), SiO_4 (H = 29.9), PO_4 (H = 17.2), NO_2 (H = 20.0) and NO_3 (H = 22.5) showed significant temporal variations (Table A.2). Water temperature was highest in August and September 2016 and lowest in January 2017. Temperatures above 17 °C were reached in the months July (III to VI) and August (I to VI) in 2015, while in 2016, the months June (IV to VI), July (V), August I to V) and September (II to VI) showed elevated temperatures (> 17 °C). Chl α peaked in June 2016 and had a sudden drop in July 2016 while generally decreasing towards the end of the year. NO_3 peaked in May 2016 and decreased towards the end of the year. SiO_4, PO_4, NO_2 and NH_4 increased towards the end of the year and showed a peak in November 2016. Temperature showed negative collinearity with DO (r = - 0.82), salinity (r = - 0.24), NO_2 (r = - 0.25), NO_3 (r = - 0.25) and with NH_4 (r = - 0.31) (Table A.3).

Occurrence of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae*

To study the spatio-temporal distribution of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* from the Elbe Estuary into the German Bight, monthly surface water samples were analyzed by species-specific MPN-PCRs. Out of 78 samples, 63 % were positive for *V. parahaemolyticus* (*Vp toxR*$^{+}$, n = 49), 40 % were positive for *V. vulnificus* (*vvhA*$^{+}$, n = 31) and 33 % were positive for *V. cholerae* (*Vc toxR*$^{+}$, n = 26) (Figure 2a).

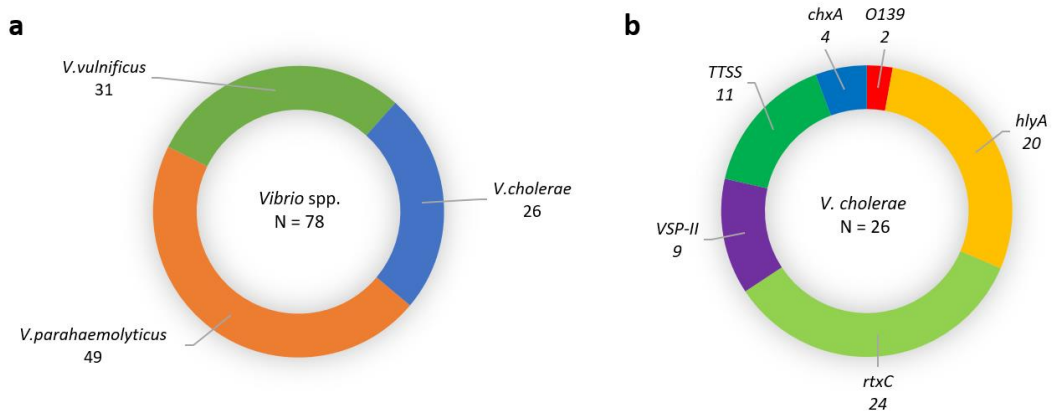

Figure 2: Number of positive samples for *Vibrio* spp. (**a**) and for *V. cholerae* virulence-associated genes (**b**) in the German Bight during the sampling campaign.

In 2015, all three species were detected from July to October (Figure 3). In 2016 on the other hand, *V. parahaemolyticus* was present from June to December, *V. vulnificus* from June to October and *V. cholerae* from May to December. *V. vulnificus* was strongly restricted to water temperatures above 13.3 °C (Figure 4). In both years, this species initially occurred after a mean threshold temperature of 17 °C was reached across the whole transect. Once present, *V. vulnificus* occurrence remained detectable even at temperatures as low as 13.3 °C, however, below this temperature this species was not detected at any sampling site.

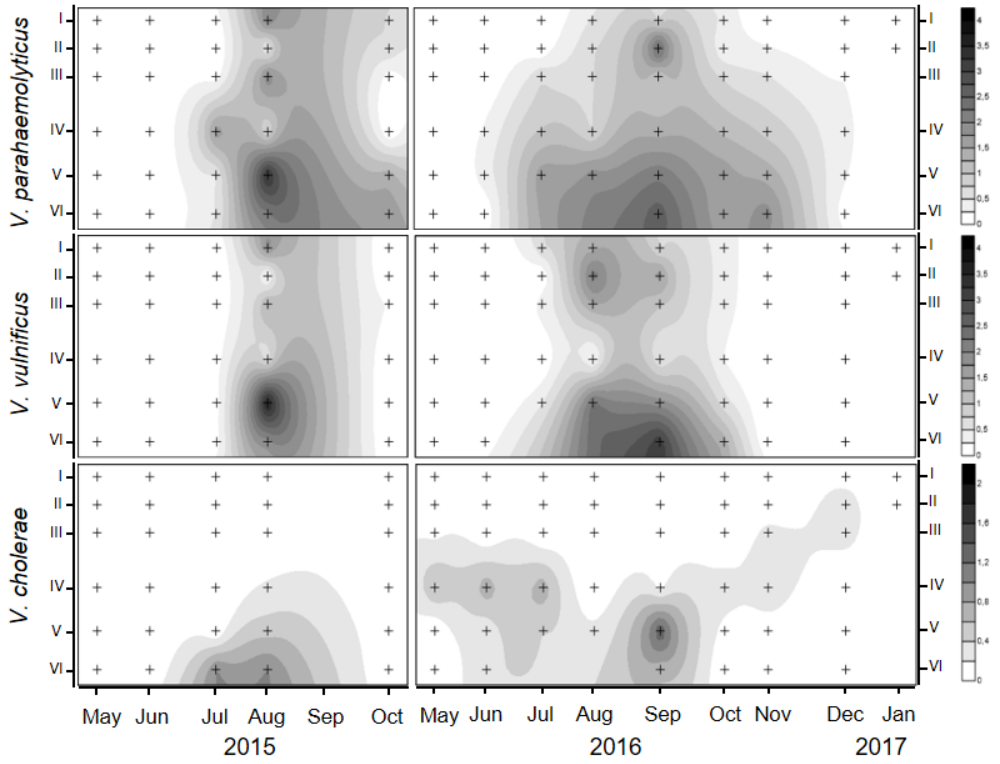

Figure 3: *Vibrio* spp. abundances [log (MPN + 1) * L^{-1}] in spatio-temporal two-dimensional space for **a**) *V. parahaemolyticus* **b**) *V. vulnificus* and **c**) *V. cholerae*. Depicted are sampling month and sampling site [I – VI]. Each cross represents one sampling point increasing color intensity depicts increasing abundance. Smoothing by kriging.

Concerning their spatial distribution, *V. parahaemolyticus* and *V. vulnificus* both occurred over the whole sampling transect while *V. cholerae* was restricted to the more coastal sites IV, V and VI in 2015. Interestingly, *V. cholerae* was detected at offshore sites (I to III) in May and December of 2016 (Figure 3).

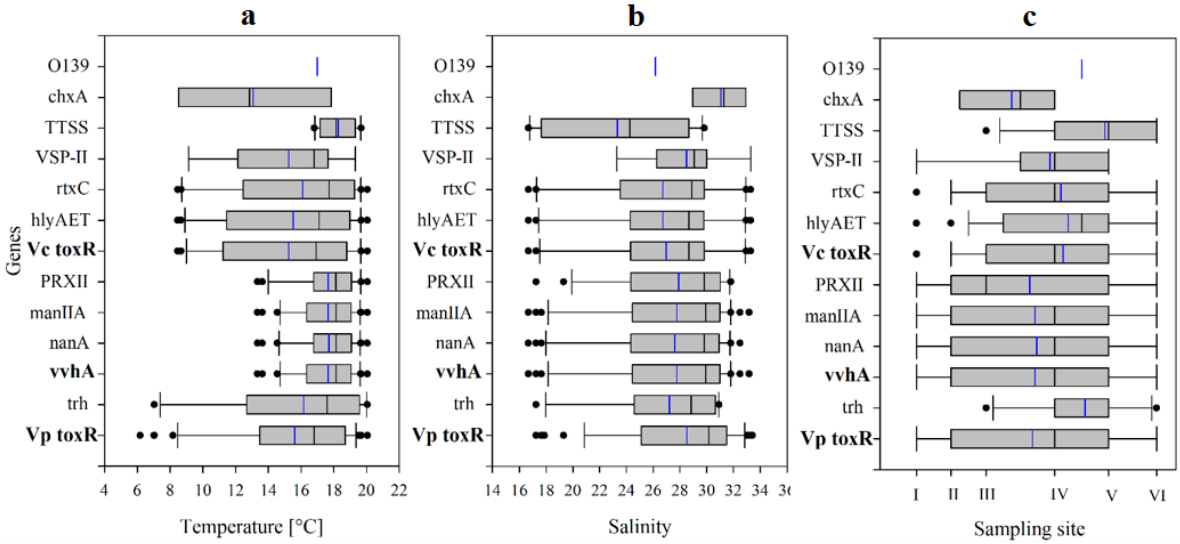

Figure 4: Species-specific and virulence-associated gene occurrence at different water temperatures (**a**), salinities (**b**) and sampling sites (**c**). Vp toxR (V. parahaemolyticus) and trh (tdh-related hemolysin). vvhA (V. vulnificus), nanA (N-acetylneuraminate lyase of the sialic acid catabolism cluster), manIIA (mannitol fermentation operon) and PRXII (Pathogenicity Region XII) analysed by the VVA1636/37 gene (PRXII^{+}). Vc toxR (V. cholerae), O139 (O139 serotype specific rfb), hlyAET (El Tor hemolysin), rtxC (Acyltransferase gene of the repeats-in-toxin cluster), chxA (Cholix Toxin), VSP-II (Vibrio 7th pandemic island II), TTSS (Type III Secretion System) analysed by the genes vcsC2, vcsN2, vspD and vcsV2. Marker genes for species-specific detection are depicted in bold. Black line depicts median; yellow line is the mean, boxes are 25th to 75th percentiles; whiskers depict minimum and maximum; dots show extrema.

Environmental predictors of *Vibrio* spp. abundances in the German Bight

All targeted *Vibrio* spp. showed higher abundances during summer months (Figure 3). *V. parahaemolyticus* abundances reached 2.4 * 10^3 MPN * L^{-1} while maximum abundance of *V. vulnificus* was 4.6 * 10^3 MPN * L^{-1} (T: 17.9 °C; S: 24.3 for both) (Figure 3, Table A.4). *V. cholerae* showed a peak of 2.4 * 10^1 MPN * L^{-1} (T: 19.3 °C; S: 24.3). High *V. parahaemolyticus* abundances of 10^2 – 10^3 MPN * L^{-1} were detected in 8 % of the samples (n = 6) (T: 17.5 – 19.6 °C; S: 17.2 – 31.3). Low *V. parahaemolyticus* abundances (10^{-1} – 10^1 MPN * L^{-1}) were detected in 55 % of the samples (n = 43) (T: 6.2 – 20.0 °C; S: 17.9 – 33.4). High *V. vulnificus* abundances (10^2 – 10^3 MPN * L^{-1}) occurred in 5 % of the samples (n = 4) (T: 17.9 – 20.0 °C; S: 17.2 – 31.4) while 35 % of the samples (n = 27) showed low *V. vulnificus* abundances (10^{-1} – 10^1 MPN * L^{-1}) (T: 13.3 - 19.7 °C; S: 16.7 - 33.2). Ninety-nine percent of the samples, where *V. cholerae* was

detected showed low abundances (10^{-1} – 10^{0} MPN * L^{-1}) (T: 8.5 – 20.0 °C; S: 16.7 – 33.3), except for one sample with maximum abundance (mentioned before).

Spearman rank order correlation revealed pronounced temporal variations of *V. parahaemolyticus* and *V. vulnificus* abundances due to collinearity with temperature (r = 0.56, r = 0.73, respectively) and DO (r = 0.44, r = 0.62, respectively) while *V. cholerae* only significantly correlated with temperature (r = 0.23) (Table A.3). Additionally, *V. cholerae* abundance showed significant correlations with salinity (r = - 0.41), cDOM (r = 0.42), SiO_4 (r = 0.25), PO_4 (r = 0.41), NO_2 (r = 0.40), NO_3 (r = 0.33), NH_4 (r = 0.30) and turbidity (r = 0.33), displaying strong spatial variations. Spatial variation was also present for *V. parahaemolyticus* and *V. vulnificus*. *V. parahaemolyticus* significantly correlated with salinity (r = -0.32), cDOM (r = 0.30), SiO_4 (r = 0.33), PO_4 (r = 0.43) while *V. vulnificus* correlated with PO_4 (r = 0.26) and with NO_3 (r = - 0.25) only.

To analyse links between abundances of the targeted *Vibrio* spp. and multiple environmental parameters, Generalized Additive Mixed Models (GAMM) were applied. Five parameters (temperature, salinity, chl α, NO_2 and NH_4) were considered as independent variables for the models after applying collinearity analysis using a variance inflation factor threshold of three (VIF = 3) which is corresponding to an $R^2 < 0.67$ in a multiple regression among the independent variables. All other environmental parameters were excluded from the modelling approach to prevent extensive multi-collinearity. The three species shared temperature and NO_2 as the main predictors for variations in abundances with non-linear patterns indicating stronger effects towards the upper range of temperatures (Figure 5). *V. parahaemolyticus* abundances were well explained by a linear model of temperature and NO_2. Abundances of *V. vulnificus* were explained by a combination of linear NO_2 and non-linear temperature terms with stronger effects at the upper range of temperatures. *V. cholerae* abundances showed a more complex dependency in a model of non-linear temperature and non-linear NO_2 terms with stronger effects at the upper range of temperatures and low NO_2 levels.

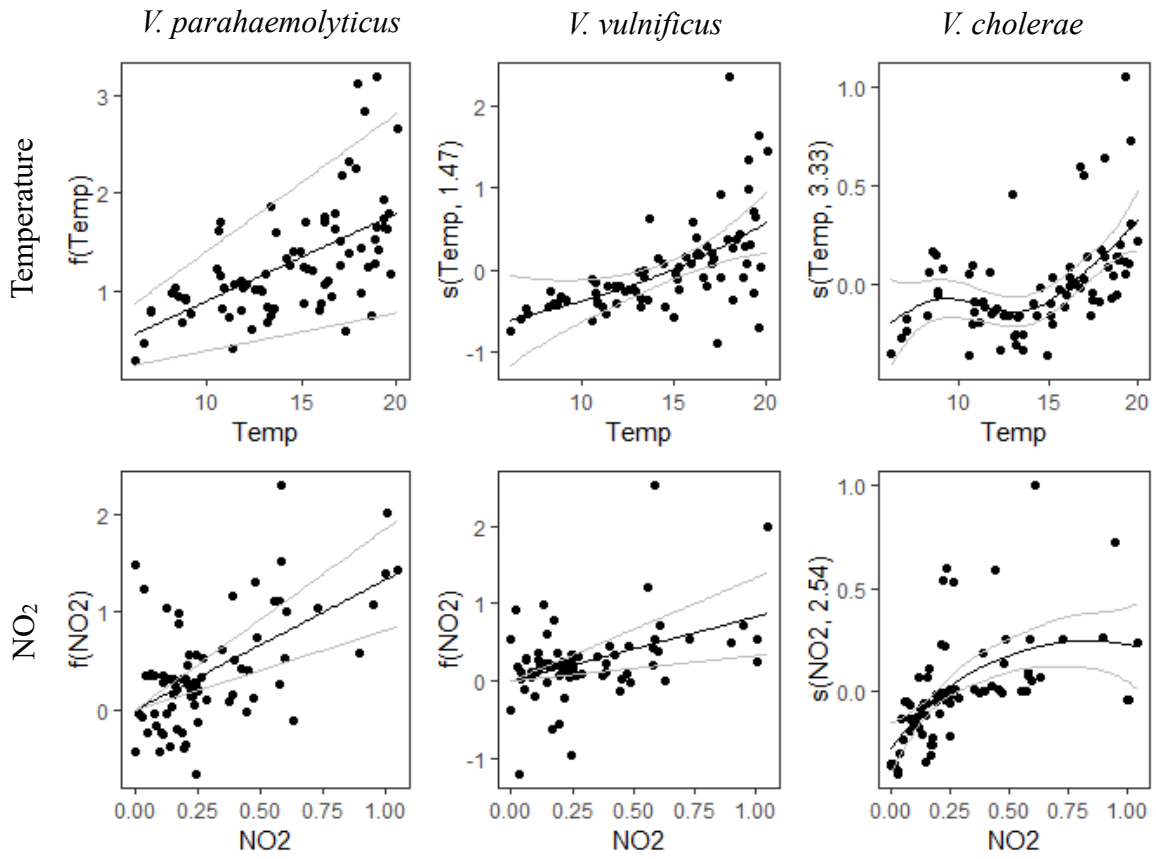

Figure 5: Fitted generalized additive models of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* abundance [log (MPN + 1) * L^{-1}] in response to the environmental parameters retained in the model; dots are the partial residuals; error bands (95%) are included. Parameters retained in the model as a non-linear term are indicated as s() or as a linear term as f().

Each model incorporated a random intercept, indicating temporal variations in abundance not taken into account by the included environmental parameters. The residual variation of *V. parahaemolyticus* and *V. vulnificus* models were highest during summer months (August and September) indicating an underestimation of high abundances by these models, while *V. cholerae* residuals were highest in December (Figure 6).

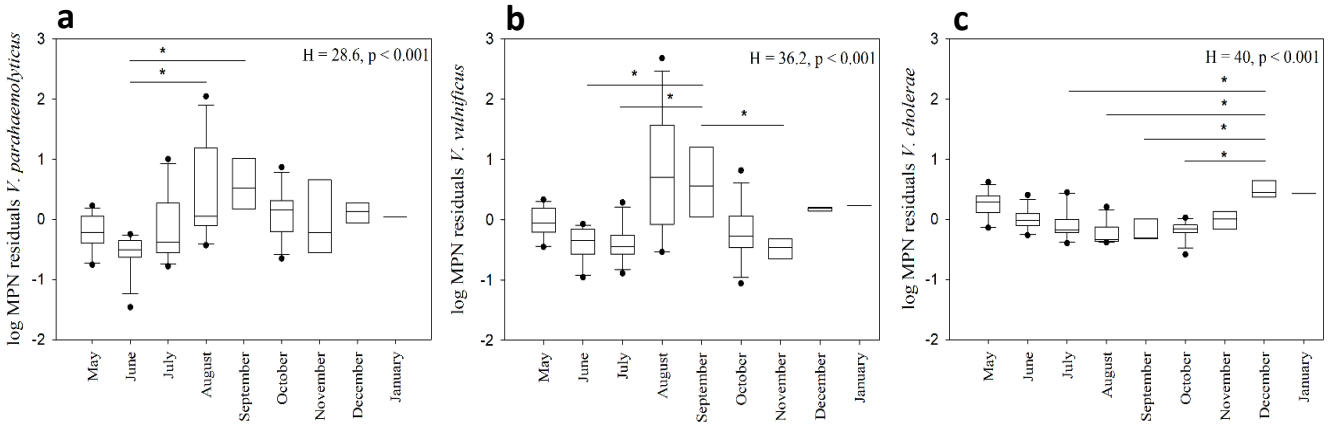

Figure 6: Log-scale normalized residuals of GAMMs per sampling month for *V. parahaemolyticus* (**a**), *V. vulnificus* (**b**) and *V. cholerae* (**c**). Horizontal lines represent the median, the boxes show the 25th to 75th percentiles, whiskers the data range and the circles represent extrema. Statistical test of residuals between months based on Kruskal-Wallis one-way ANOVA (H) statistics followed by Dunn post-hoc tests for pairwise comparisons. Pairs significantly different at a level of $p < 0.05$ are depicted by asterisk.

Virulence-associated genes of *Vibrio* spp.

***V. parahaemolyticus:* Tdh and trh**

Tdh was not detected at any sampling site or time. However, in 40 % of the *Vp toxR*$^{+}$ samples *trh* was detected (n = 10) (Table A.4) in a temperature range of 7 – 20 °C and at salinities between 17.2 and 30.9 mainly at coastal sites (Figure 4). Mann-Whitney U test indicated solely a correlation between *trh* occurrence and *V. parahaemolyticus* abundance (MPN * L^{-1}) (U = 111; $p < 0.05$) (Table A.5).

***V. vulnificus*: NanA, manIIA and PRXII**

ManIIA was detected in all *vvhA*$^{+}$ samples while the majority carried additionally the *nanA* gene (96 %) (Table A.4). PRXII was present in 74 % of all *vvhA*$^{+}$ samples (PRXII^{+}, n = 23) while in 71 % this genomic island (PRXII^{-}) was absent. DNA sequencing of the *VVA1636/37* (PRXII^{+}) PCR fragment confirmed the presence and identity of PRXII (ca. 98 % identity). Presence of all three virulence-associated genes was detected along the whole sampling transect and the entire temperature range of *vvhA*$^{+}$ (13.3 – 20°C), while their salinity ranges differed slightly in

the upper range (*vvhA*$^+$ & *manIIA*$^+$: 16.7 – 33.2; *nanA*$^+$: 16.7 – 32.5, PRXII$^+$: 17.2 – 31.8) (Figure 4). Mann Whitney U Tests revealed no significant differences in the occurrence of PRXII$^+$ in relation to any observed environmental parameter or *V. vulnificus* abundance (Table A.5). A test for significance was obsolete for *manIIA* and *nanA*, as nearly all samples were positive for these genes.

Three different virulence profiles (VvP-1 – 3) were detected, composing a combination of the investigated virulence-associated genes. The majority of samples (74 %) contained all targeted virulence-associated genes (VvP-3, n = 23) while one sample carried the profile VvP-1 (*vvhA*$^+$*manIIA*$^+$) (3 %) and seven samples (23 %) carried the profile VvP-2 (*vvhA*$^+$*manIIA*$^+$*nanA*$^+$) (Table A.6).

***V. cholerae:* O1 & O139 and ctxA, tcpAET, rtxC, hlyAET, VSP-II, TTSS and chxA genes**

The pandemic *V. cholerae* serotype O1 and the two major virulence factors *ctxA* and *tcpAET* were entirely absent from *Vc toxR*$^+$ samples. However, the pandemic serotype O139 was present in two samples at 16.8 °C and 17.2 °C and at salinities of 23.3 and 29 close to the coast (Figure 4). Presence and identity of the serotype O139 was confirmed by sequencing of the PCR amplicons (ca. 99 % identity). Mann Whitney U test revealed no significant correlation between presence of serotype O139 and any environmental parameter or *V. cholerae* abundances (Table A.5).

For further detailed characterization of *Vc toxR*$^+$ samples, the presence of a number of accessory virulence-associated genes was examined. Gene occurrence frequencies are displayed in figure 2b while figure 4 shows occurrence with regard to temperature, salinity and sampling site. *RtxC* was detected in 92 % (n = 24) and *hlyAET* in 77 % (n = 20) of *Vc toxR*$^+$ samples covering the entire temperature and salinity ranges (T: 8.5 – 20 °C; S: 16.7 – 33.3) without a general spatial or temporal trend of occurrence. *VSP-II* was targeted with primers revealing the absence of this genomic island. The samples that showed no PCR product were not further analysed for genetic composition or completeness of the genomic island but were assumed to harbour *VSP-II*. *VSP-II* was present in 35 % of *Vc toxR*$^+$ samples (n = 9) at temperatures between 9.1 and 19.3 °C and at salinities between 23.3 and 33.3 mainly located close to the coast. Forty-two percent (n = 11) of the *Vc toxR*$^+$ samples were found to contain

TTSS with all four targeted genes present (*vcsC2*, *vcsN2*, *vspD* and *vcsV2*). Interestingly, TTSS occurred at a narrow temperature range of 16.8 – 19.7 °C and at salinities from 16.7 – 30 but mainly at coastal sites. Four offshore samples contained *chxA* (15 %), which appeared within a wide temperature range of 8.5 – 17 °C and a salinity range of 28.7 – 33.

Statistical validation of environmental dependencies by Mann Whitney U Tests (Table A.5) showed no significant correlation of *VSP-II* and *chxA* occurrence with environmental parameters or with *V. cholerae* abundance. However, *rtxC* correlated significantly with PO_4 levels ($U = 2$; $p < 0.05$) and *hlyAET* correlated significantly with NH_4 levels ($U = 23$; $p < 0.05$). Interestingly, several significant correlations were detected between TTSS and *V. cholerae* abundance (MPN * L^{-1}) ($U = 21$; $p < 0.001$), salinity ($U = 21$; $p < 0.001$), NO_3 ($U = 24$; $p < 0.01$), temperature ($U = 25$; $p < 0.01$), turbidity ($U = 33$; $p < 0.01$), PO_4 ($U = 36$; $p < 0.05$) as well as Chl α ($U = 42$; $p < 0.05$).

V. cholerae virulence profiles

To unravel changes in the spatio-temporal composition of the *V. cholerae* community, the combinations of virulence-associated genes per sample were analysed. *V. cholerae* samples showed 12 different virulence profiles (VcP-1 – 12) revealing a high genetic variation among *V. cholerae* samples with two to six co-occurring virulence-associated genes (Table A.7). Most samples with four to six co-occurring virulence-associated genes appeared close to the coast (Figure 7) above 16.8 °C. The genes TTSS, *chxA* and O139 were detected only in co-occurrence with other virulence-associated genes. The serotype O139 occurred in combination with *hlyAET*, *rtxC*, *VSP-II* and TTSS genes (VcP-12) exclusively, pointing to the possible presence of a high-risk virulence profile. The profile VcP-5 (*toxR*$^+$*rtxC*$^+$*hlyAET*$^+$) was present in four *Vc toxR*$^+$ samples (15 %) and showed the broadest temperature range (11.8 – 20 °C) but a narrow salinity range (28.6 – 29.8). The profiles VcP-6 (*toxR*$^+$*rtxC*$^+$*hlyAET*$^+$*VSP-II*$^+$) and VcP-7 (*toxR*$^+$*rtxC*$^+$*hlyAET*$^+$*chxA*$^+$) were only detected at offshore sampling sites (salinity: 32.8 – 33.3) at temperatures between 8.5 and 9.1 °C. The most frequently occurring profile VcP-8 (*toxR*$^+$*rtxC*$^+$*hlyAET*$^+$TTSS$^+$) was present in 27 % of *Vc toxR*$^+$ samples (n = 7), being the only detected profile at the sampling site VI located closest to the coast. This virulence profile spans the broadest range of salinity (16.7 – 29.8) but is restricted to temperatures above 17 °C. All

other virulence-associated genes occur with varying spatio-temporal distributions showing no apparent pattern.

		2015				2016							
		July	Aug	Sept	Oct	May	June	July	Aug	Sept	Oct	Nov	Dec
Offshore	I			N/A									- - - *VSP-II* *hlyAET* *rtxC*
	II			N/A		- - - - *hlyAET* -							- *chxA* - - *hlyAET* *rtxC*
	III			N/A		- - - - *hlyAET* *rtxC*				- - *TTSS* - *hlyAET* *rtxC*		- - - *VSP-II* - *rtxC*	- *chxA* - - *hlyAET* *rtxC*
	IV		- *chxA* - *VSP-II* *hlyAET* *rtxC*	N/A		- - - *VSP-II* - -	- *chxA* *TTSS* *VSP-II* *hlyAET* *rtxC*	*O139* - *TTSS* *VSP-II* *hlyAET* *rtxC*		- - - - - *hlyAET* *rtxC*	- - - - - *hlyAET* *rtxC*	- - - - - *rtxC*	
Coastal	V		- - *TTSS* - *hlyAET* *rtxC*	N/A	- - - *VSP-II* - *rtxC*		- - *TTSS* - *hlyAET* *rtxC*	*O139* - *TTSS* *VSP-II* *hlyAET* *rtxC*	- - - *hlyAET* *rtxC*	- - *TTSS* *VSP-II* *hlyAET* *rtxC*	- - - - *rtxC*	- - - - *rtxC*	
	VI	- - *TTSS* - *hlyAET* *rtxC*	- - *TTSS* - *hlyAET* *rtxC*	N/A			- - *TTSS* - *hlyAET* *rtxC*	N/A	N/A	- - *TTSS* - *hlyAET* *rtxC*			

Figure 7: Spatio-temporal distribution of *V. cholerae* virulence-associated gene combinations (virulence profiles) in the Elbe transect of the German Bight. *Vc toxR* is omnipresent and excluded from this figure. *O139* (O139 serotype specific rfb), *hlyAET* (El Tor hemolysin), *rtxC* (Acyltransferase gene of the repeats-in-toxin cluster), *chxA* (Cholix Toxin), *VSP-II* (Vibrio 7th pandemic island II), *TTSS* (Type III Secretion System) analysed by the genes *vcsC2, vcsN2, vspD* and *vcsV2*. *Vc toxR*-negative samples were not analysed for virulence-associated genes and are depicted in grey. N/A are not available samples.

Discussion

Extended warm period coincides with prolonged *Vibrio* spp. occurrence

In surface waters of the German Bight, all three *Vibrio* species, *V. parahaemolyticus, V. vulnificus* and *V. cholerae* were present at each sampling site from the Elbe estuary to the island of Helgoland (Figure 1). *Vibrio vulnificus* prefers brackish waters, usually inhabiting waters below a salinity of 24 and is most commonly detected between 8 – 16 (Jacobs et al., 2014), with occasional occurrences at higher salinities (30) (Böer et al., 2013). In this study, *V. vulnificus* was detected close to Helgoland (salinity up to 33) during summer months in both years. These results support the hypothesis that *V. vulnificus* can occur at a broader salinity range when high temperatures prevail (Randa et al., 2004). *V. cholerae* occurred with a spatial preference for coastal sites occurring generally in low abundances, as reported previously for the German Bight (Böer et al., 2013). This species emerged at offshore sites at lower temperatures in spring and fall of 2016.

The three targeted potentially pathogenic *Vibrio* species are commonly detected in warmer waters, especially when water temperatures rise above 17 °C (Oliver et al., 2013). In the present study, *V. parahaemolyticus* and *V. cholerae* were both detected at temperatures of 6.2 and 8.5 °C in low abundances, respectively showing tolerance to low temperatures. *Vibrio vulnificus* has a temporally limited occurrence period suddenly appearing and disappearing with a restriction to temperatures above 13.3 °C. This species requires elevated temperatures for annual first occurrence, which has already been shown for the German Bight. This previous study based on isolation stated that a threshold of 20 °C has to be reached for sufficient population growth to be able to detect the sudden appearance of *V. vulnificus* at recreational beaches of the German Bight (Böer et al., 2013). In the present study, the mean temperature of the first *V. vulnificus* detection during the season was much lower (17.9 °C). Once detected, *V. vulnificus* remained present for a long period of time and was detectable at significantly lower temperatures (13.3 °C), as demonstrated elsewhere (Baker-Austin and Oliver, 2018, Böer et al., 2013).

The North Sea is predicted to be one of the most suitable areas for the incidence of *V. cholerae* under current and future climate scenarios (Escobar et al., 2015). Our observations assessed different occurrences for all three *Vibrio* spp. with respect to the different years. The earlier

occurrence and prolonged duration of *V. parahaemolyticus, V. vulnificus* and *V. cholerae* in 2016 coincides with the earlier warming of surface waters and longer time window with seawater temperatures above 17 °C (four months: June to September 2016). Similarly, extended warm periods have been shown to provide a basis for longer occurrences of viable potentially pathogenic *Vibrio* spp. in the Sydney Harbour estuary, Australia (Siboni et al., 2016). Also in other marine habitats i.e. molluscan shellfish beds in a temperate east Canadian estuary, an increased frequency of potentially pathogenic *Vibrio* spp. detection was concurrent with ongoing seawater warming conditions over a study period of 11 years (Banerjee et al., 2018). The trend of extended warm periods has been identified for the whole North Sea (Vezzulli et al., 2016) and is estimated to increase further with climate change (Brennholt et al., 2014). A climate model projection of sea surface temperature within the scope of the German research program KLIWAS predicted an increase in the number of months with SST > 17 °C from 2 - 3 (1970 - 1999) to 3.5 - 4.5 months in the near future (2021 - 2050) for the North Sea (Brennholt et al., 2014). This likely will further expand the spatio-temporal extent of these potentially pathogenic *Vibrio* species presumably accompanied by an increased exposure and infection risk in the German Bight.

Vibrio spp. abundances respond to temperature and nutrients in the German Bight

Spatio-temporal fluctuations of abundances were observed for the three targeted *Vibrio* spp.. They showed significant temporal variations with increasing abundances during summer months, similar to earlier observations (Böer et al., 2013, Davis et al., 2017). Observed maximum abundances of all three species are in an expected range, according to a previous study at German recreational beaches based on isolation (Böer et al., 2013) and in a French coastal lagoon based on real-time PCR analysis (Esteves et al., 2015a). *Vibrio parahaemolyticus* and *V. vulnificus* peaked during an event of a seawards shifted salinity gradient in summer 2015 together with elevated nutrient levels, indicating an increased freshwater discharge event from the river Elbe (Voynova et al., 2017). This incident is underpinned by previous studies reporting rapid proliferation of potentially pathogenic *Vibrio* spp. as a consequence of heavy river discharge into a Mediterranean lagoon after freshwater flash floods (Esteves et al., 2015a) and in response to the Hurricane Matthew in the Neuse River Estuary, North Carolina (Jesser and Noble, 2018).

Seawater warming and heatwave events are common in northern European waters, these incidences are further expected to increase, especially in the North Sea, a region which already underwent a significant warming from 1962 to 2008 by an average of 1.67 °C (Wiltshire et al., 2010). Surface water in this region occasionally reach temperatures in which potentially human pathogenic *Vibrio* spp. are likely to occur (> 17 °C) (Oliver et al., 2013, Escobar et al., 2015). In the present study however, some of these samples (> 17 °C) were tested negative for *Vibrio* spp. With this, an indication of a more complex environmental dependency is suggested, shown by the significant concomitant associations of potentially pathogenic *Vibrio* spp. abundances with temperature and NO_2. The correlations between the abundances of all three species with temperature (Spearman and GAMM) supporting their strong seasonality, are in concordance with a multiple linear regression model of *Vibrio* abundances on a genus level on Helgoland Roads (Oberbeckmann et al., 2011b). Accordingly, another study assumed water temperature to be the most important factor dictating *V. parahaemolyticus* and *V. vulnificus* abundances in recreational beaches of the German Bight (Böer et al., 2013). While *V. parahaemolyticus* has a linear relationship to temperature in the present study, *V. vulnificus* and *V. cholerae* showed non-linear responses with greater associations at elevated temperatures. This finding is in agreement with correlations between extreme events like heatwave incidences and *Vibrio* infection cases in temperate regions (Baker-Austin et al., 2016). Our GAMMs show that increased temperature is a crucial driver of elevated abundances of each *Vibrio* spp.. Although this is supported by previous studies (Vezzulli et al., 2013, Vezzulli et al., 2016), temperature cannot explain spatio-temporal *Vibrio* abundance patterns in the German Bight alone.

We found associations of potentially pathogenic *Vibrio* spp. abundances, especially of *V. cholerae,* with NO_2 levels. Regarding the inorganic nitrogen metabolism, *V. cholerae* was reported to reduce NO_3 to NO_2 under hypoxic alkaline conditions (Bueno et al., 2018) or under conditions of glucose limitation (Macfarlane and Herbert, 1982). Yet, these environmental conditions (hypoxic alkaline or glucose limitation) are not prevalent in the present study. However, physicochemical parameters show strong correlations, as previously reported for the German Bight (Oberbeckmann et al., 2012, Lucas et al., 2016). For achieving low multi-collinearity, the parameters PO_4, NO_3, SiO_4, DO, cDOM and turbidity were excluded from the models. However, given the significant linear correlations between NO_2, salinity and other nutrients, indirect effects of these cannot be excluded and multiple factors could be involved

in the generation of a favourable environment for the growth of *Vibrio* species. Considering this, we assume that our finding of NO_2 being a good predictor for *Vibrio* abundances, might be better explained as proxy for collinear parameters and is in agreement with studies reporting high nutrient loads as drivers of spatial variations and elevated *Vibrio* abundances (Böer et al., 2013, Oberbeckmann et al., 2011b).

Diverse virulence patterns of pathogenic *Vibrio* spp. in the German Bight

Trh* positive *V. parahaemolyticus

Vibrio parahaemolyticus strains, that have the ability to produce the thermostable direct hemolysin (*tdh*) or the tdh-related hemolysin (*trh*) are considered as pathogenic strains (Nair et al., 2007). Pathogenic *V. parahaemolyticus* strains were not present in the marine environment around Helgoland in 2010 (Oberbeckmann et al., 2011b) but were later detected at coastal recreational beaches of the German Bight in few bacterial isolates (Böer et al., 2013). Previous studies, mostly based on bacterial isolates, showed that generally only few (< 5 %) environmental *V. parahaemolyticus* isolates carry either the *trh* or the *tdh* gene (Lopatek et al., 2018, DePaola et al., 2000, DePaola et al., 1990). Our study supports the assumption, that the presence of the *trh* gene accompanied by the absence of *tdh* is a common characteristic shared by northern European *V. parahaemolyticus* populations (Ellingsen et al., 2008, Böer et al., 2013). *Trh* was present in the entire sampling transect (coastal to offshore) and in the whole temperature range of this study (7 - 20 °C) but did not show significant associations with any environmental parameters indicating to a good environmental adaptation. On the other hand, the trh^+- population linearly correlated with *V. parahaemolyticus* abundances in the German Bight. In contrary to this, several studies showed that high total *V. parahaemolyticus* counts do not necessarily indicate that pathogenic subpopulations are more likely to be present (Phippen et al., 2017, Julie et al., 2010).

Presence of pathogenic *V. vulnificus*

Environmental *V. vulnificus* strains from coastal waters of the German North Sea possess a higher virulence potential than these from coastal waters of the German Baltic Sea (Bier et al., 2015). Yet, death and infection cases are documented regularly and exclusively for the Baltic Sea (Frank et al., 2006, Huehn et al., 2014, Baker-Austin et al., 2013), while only sporadic infections were reported in the North Sea region so far (Huehn et al., 2014). In this study, the *V. vulnificus* virulence-associated genes *nanA*, *manIIA* and PRXII did not reveal any spatio-temporal pattern and were nearly omnipresent whenever *V. vulnificus* was detected, posing a possible infection threat throughout the summer even at low *V. vulnificus* abundances.

Evidence for epidemic *V. cholerae* serotype O139

For the first time we proved the presence of nontoxigenic *V. cholerae* O139 serotype in the German Bight, where epidemic serotypes had not been detected before. A possible explanation for its presence could be that environmental nontoxigenic *V. cholerae* may have acquired the genetic cassette responsible for biosynthesis of the O139 surface polysaccharide (Zhang et al., 2014). However, rather than the genetic acquisition, a localized transport event of epidemic *V. cholerae* strains into this region is a more likely explanation due to a spatio-temporal limited O139 detection in this study. Connecting two of the largest ports in the North Sea region (Hamburg and Bremerhaven) with the world (van der Meer et al., 2016), the Elbe estuary is accordingly exposed to extensive shipping traffic. The transport and discharge of potentially pathogenic *Vibrio* spp. (Ng et al., 2018) and especially of epidemic *V. cholerae* strains (Dobbs et al., 2013) by shipping traffic has been shown previously. Relevantly, the discharge of toxigenic *V. cholerae* O1-containing ballast water is believed to have caused the *V. cholerae* pandemic in Latin America in 1991 under favourable seawater conditions during an El Nino event (Seas et al., 2000). Despite the recently adopted Ballast Water Management (BWM) convention by the International Maritime Organization (IMO) to reduce the introduction of harmful organisms into new environments, several publications point to the fact that most treatments do not reduce the risk of pathogen dispersal and that regrowth after ballast water treatment is common (Petersen et al., 2019, Hess-Erga et al., 2010).

The two major virulence factors of toxigenic *V. cholerae* serotypes O1 and O139, the Cholera toxin (CT) and toxin co-regulated pilus (TCP), are scarce in northern temperate waters (Böer et al., 2013, Schuster et al., 2011, Brennholt et al., 2014, Schwartz et al., 2019) and concordantly, all *V. cholerae* samples in this study are non-toxigenic (*ctxA*$^{-}$*, tcpAET*$^{-}$).

In addition to these previously discussed virulence factors, several accessory virulence-associated genes are commonly targeted for the assessment of potential pathogenicity and for genetic characterization of clinical and environmental non-O1/non-O139 *V. cholerae* strains (Abia et al., 2017, Hasan et al., 2013, Ceccarelli et al., 2015, Schirmeister et al., 2014). The *V. cholerae hlyAET* gene is a pore-forming cytotoxin that induces vacuolation of eukaryotic host cells and ultimately leads to cell lysis (Figueroa-Arredondo et al., 2001). *RtxC*, the acyltransferase of the RTX cluster (Lin et al., 1999), is an activator of the multifunctional autoprocessing repeats-in-toxin (MARTX) cytotoxin gene (*rtxA*). This study showed frequent *hlyAET* and *rtxC* occurrence without any general spatial or temporal trend. *HlyAET* presence as well as its haemolytic activity in *V. cholerae* was detected in the majority of isolates previously screened in the North and Baltic Sea, while the *rtxC* gene was present in all tested *V. cholerae* isolates (Schwartz et al., 2019).

The contribution of *rtxC* to MARTX-associated virulence is still unclear since the *rtxC* gene is not required for its cytotoxicity (Cheong et al., 2010, Satchell, 2007). Yet, both genes were also present in previous clinical isolates from Germany (Schwartz et al., 2019). The Vibrio 7th pandemic islands (VSP-I & II) were acquired by the 6th pandemic O1 classical strains via horizontal gene transfer and provided increased environmental fitness to these strains. These 7th pandemic islands were responsible for the success of the 7th pandemic O1 El Tor strains, thereby entirely replacing the classical strains (Dziejman et al., 2002). Hence, VSP-II is commonly found in toxigenic strains and in contrast is rarely detected in clinical and environmental non-O1/non-O139 strains (Chatterjee et al., 2009, Rahman et al., 2008, Schirmeister et al., 2014). In this study, VSP-II was present in environmental non-toxigenic *V. cholerae* mainly at coastal sites and additionally at offshore sites in winter. A clear contribution of VSP-II to virulence in humans is not elucidated but it has been reported that the presence of this genomic island might confer a selective advantage to toxigenic *V. cholerae* in the human host (Taviani et al., 2010). The type III secretion system (TTSS) is an apparatus to secrete and deliver virulence factor proteins directly into eukaryotic host cells (Hueck, 1998, Okada et al.,

2009, Park et al., 2004). This study showed frequent TTSS presence (42 %) and additionally elucidated an association of TTSS to warm temperatures and coastal sites where elevated *V. cholerae* abundances occurred. Generally, TTSS is broadly distributed among environmental and clinical non-O1/non-O139 *V. cholerae* strains (Dziejman et al., 2005, Rahman et al., 2008) while in contrast, only few environmental isolates showed TTSS occurrence (7 %) in the German North and Baltic Sea (Schwartz et al., 2019). TTSS has been demonstrated to enhance the virulence of clinical non-O1/non-O139 *V. cholerae* strains (Zeb et al., 2019), causing death even more rapidly than toxigenic TCP^+ *V. cholerae* strains in infant rabbit experiments (Shin et al., 2011). In German and Austrian patients infected with non-O1/non-O139 *V. cholerae*, TTSS was detected in all diarrheal cases while being absent from other contact-based (otitis, wound infection) infection cases (Schirmeister et al., 2014, Schwartz et al., 2019). Another accessory virulence trait, the Cholix Toxin (*chxA*), is the third member of the diphtheria toxin group, a mono-ADP-ribosyltransferase toxin, preferentially occurring in non-O1/non-O139 clinical and environmental *V. cholerae* strains (Purdy et al., 2010, Schirmeister et al., 2014, Awasthi et al., 2013). C*hxA* is capable of inhibiting protein synthesis in eukaryotic cells and ultimately leading to cell death (Jørgensen et al., 2008) and is suggested to be associated with human extra-intestinal infections (like septicaemia) rather than enterotoxicity (Awasthi et al., 2013). In the present study, *chxA* was detected in lower frequencies, corresponding with a previous study (Schwartz et al., 2019), but showed a restriction to high salinities and a broad temperature range, suggesting that this gene contributes to the survival of *V. cholerae* under semi-optimal conditions in offshore regions. The accessory virulence-associated traits of *V. cholerae*, in particular the hemolysin, RTX cluster, Type III Secretion System and the *Vibrio* 7^{th} pandemic island, which have been suggested to play a role in environmental fitness (Rahman et al., 2008, Dziejman et al., 2005, Sakib et al., 2018, Satchell, 2015), may provide selective advantage to emerging pathogenic *V. cholerae* strains. Although our results suggest a similar phenomenon of increased environmental fitness by certain accessory virulence-associated genes, *V. cholerae* populations in the German Bight need to be examined further in genomic studies on a spatio-temporal basis.

Divergent high-risk virulence profiles of *V. cholerae*

Recently, *V. cholerae* isolates comprising diverse virulence profiles within environmental samples have been detected globally, indicating a dynamic *V. cholerae* community at ecotype levels (Ceccarelli et al., 2015, Gong et al., 2018, Keymer et al., 2007, Schwartz et al., 2019). Previous studies have shown the presence of unconventional virulence gene combinations in clinical (Nair et al., 2002, Ansaruzzaman et al., 2004) and environmental (Ceccarelli et al., 2015) *V. cholerae* strains, demonstrating the genetic plasticity of this species. Epidemic *V. cholerae* strains evolved from environmental non-pathogenic strains by acquisition of virulence genes through lysogenic bacteriophages (CTXPhage; Cholera Toxin) or through genetic elements transported through horizontal gene transfer (HTG) (Mekalanos, 2012, Hazen et al., 2010). Given the genetic plasticity of environmental *V. cholerae* and the fact that most virulence factors are encoded on mobile genetic elements, any strain is theoretically able to become a pathogen (Kirchberger et al., 2016, Gong et al., 2018). Gong et al. (2018) showed via whole genome sequencing of non-O1/non-O139 strains that *V. cholerae* is able to undergo extensive genetic recombination via HGT during its evolution. In this context, Hammerl et al. (2017) recently detected the antimicrobial trait carbapenemase acquired by environmental non-toxigenic, non-O1/non-O139 *V. cholerae* strains in German coastal waters in 2015.

Our observations show an increased co-occurrence in virulence-associated genes per sample during summer months with profiles consisting of more than four virulence-associated genes at temperatures above 16.8°C. This demonstrates an increased diversity as water temperature increases, as previously shown for *V. parahaemolyticus* populations in New Hampshire estuary (Great Bay Estuary) (Ellis et al., 2012). Esteves et al. (2015c) showed high recombination activity of *V. cholerae* in French coastal regions, especially in high-salinity conditions (> 20 ppt). The level of genetic diversity of *V. cholerae* on the other hand is reported to be higher at low salinity and high temperature conditions (Esteves et al., 2015c).

The serotype O139 occurred only in the VcP-12 profile (*toxR*$^{+}$*rtxC*$^{+}$*hlyAET*$^{+}$*VSP-II*$^{+}$TTSS^{+}*O139*$^{+}$, Table A.7), representing a high-risk virulence profile. The virulence profiles VcP-6, VcP-7, VcP-8 and VcP-10 (Table A.7) of this study, including other additional virulence-associated genes (*dth* and *rtxA*), have previously been detected in clinical strains originating from diarrheal and otitis infection cases in Germany and Austria (Schirmeister et al., 2014). The infection cases were mostly travel-associated and imported to these European countries; however, the

present study again demonstrates the possibility of infection risks originating locally due to the presence of pathogenic *V. cholerae* in the German Bight.

Considering the high spatio-temporal variability of virulence profiles in this study, it is striking that in station VI , only one profile (VcP-8; *toxR*$^{+}$*rtxC*$^{+}$*hlyAET*$^{+}$TTSS^{+}) was found over the entire study period, probably indicating a highly adapted *V. cholerae* population to the estuary at the Elbe river mouth. Recently, Gong et al. (2018) discovered distinct estuary environment-specific genes of non-O1/non-O139 *V. cholerae* strains in the Yangtze River estuary in China via genome analysis. Their comparative genomic studies among strains from Yangtze River estuary and environmental *V. cholerae* strains from other ecosystems revealed 28 unique genes. The authors propose that a diverse set of genes display a unique environmental adaptation of *V. cholerae* to the estuary (Gong et al., 2018), supporting the results of this study. Given the co-occurrence of multiple virulence-associated genes per samples in this study, an emergence and establishment of new pathogenic strains in the German Bight is likely possible.

Conclusion

German waters currently lack systematic surveillance for potentially human pathogenic environmental *Vibrio* spp. This study expands the limited existing information on the spatio-temporal distribution, abundance and pathogenic characteristics of the three relevant potentially pathogenic *Vibrio* species, *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* in the German Bight. The species-specific quantification using the method MPN-PCR enabled a detailed analysis of *Vibrio* variability, both qualitatively and quantitatively. Future studies should include metagenomics approaches to environmental samples to assess the ecotype diversities of potentially pathogenic *Vibrio* species. Of particular interest would be the question of whether specific environmental conditions may favour *Vibrio* strains with greater infective risk. High temporal and spatial resolution studies would help to elucidate community shifts of *V. cholerae* in the German Bight in the future, allow authorities to predict, detect, and mitigate, any public health risks in advance. *Vibrio cholerae* serotype O139 occurrence in particular, as well as the highly diverse virulence profiles detected, highlight the importance of the need for continuous monitoring by public authorities for the presence and spatio-temporal changes of *Vibrio* spp. virulence-associated genes. This is especially the case for human high-risk exposure locations like coastal recreational beaches. We predict that climate change related events such as the expected further rise in seawater temperatures combined with extended warm periods and increased nutrient concentrations from river discharge after heavy rainfall events would create a favourable environment for human pathogenic *Vibrio* spp. in the German Bight. In future, this environmental shift is likely to support an increased occurrence of infections by direct contact with contaminated water or by ingestion of contaminated food in this region.

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Chapter II

In vitro growth potentials of clinically relevant *V. cholerae* and *V. vulnificus* strains in a salinity gradient in the German Bight (North Sea)

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Abstract

Human pathogenic *Vibrio* spp. are becoming increasingly significant in northern European waters. The majority of *V. cholerae* and *V. vulnificus* populations distributed throughout the southeastern North Sea (German Bight) were found to be of potential clinical relevance due to the presence of multiple virulence factors. However, little is known about the specific *in situ* environmental conditions that control the growth of those *Vibrio* strains in the marine environment. This study investigated the potential of a broad range of physicochemical conditions to support the growth of clinically relevant *Vibrio* spp. strains along a salinity gradient of the German Bight. For that, pathogen growth potential (PGP) assays have been conducted with *V. vulnificus* (vcg type C*, nanA*$^{+}$*, manIIA*$^{+}$) and *V. cholerae* (VSP-II^{+}*, chxA*$^{+}$*, hlyAET*$^{+}$*, rtxC*$^{+}$), possessing multiple virulence associated genes. *In vitro* growth assays were conducted to stationary phase in sterile seawater at *in situ* temperature (realized niche, flow cytometry) and in a broad salinity-temperature range at high-nutrient growth medium (fundamental niche, spectrophotometry). *V. vulnificus* and *V. cholerae* strains could grow in most seawater samples and growth kinetics of both species showed strong temperature dependency with a particular acceleration of growth above 17.5°C. Population density of *V. vulnificus* was temperature dependent while it was driven by nitrogen concentrations for *V. cholerae* population density. Interestingly, a dependency towards salinity was only found for the population density of both species under high-nutrient conditions. Furthermore, we could demonstrate that nutrient availability is significantly changing growth response of both *Vibrio* spp. towards salinity and temperature. We conclude that clinically relevant *V. vulnificus* and *V. cholerae* species are able to spread in the German Bight under ambient physicochemical conditions. Considering the North Sea as one of the fastest warming seas worldwide, routine surveillance studies are strongly recommended for the German Bight in future. To our knowledge, this is the first attempt of a microbial risk assessment approach of clinically relevant *Vibrio* strains in the German Bight and serves as an important baseline for all future surveillance studies.

Introduction

Vibrio vulnificus and *Vibrio cholerae* are ubiquitous marine bacteria, autochthonous to estuaries and coastal waters. Worldwide, those species are also known as water-related human pathogens able to cause serious gastroenteritis, wound infections or septicaemia (Thompson et al., 2004a). *V. cholerae* strains of the O1 or O139 serogroups are the causative agents of the Cholera, especially those carrying the cholera toxin (CT) and toxin-coregulated pilus (TCP). Generally, *V. vulnificus* strains which possess the 16S rRNA - type B allele and the virulence correlated gene (vcg) type C, are considered as pathogenic (Rosche et al., 2005, Vickery et al., 2007). However, some studies showed a high association of additional genes with strains originating from clinical cases from the Baltic Sea and the northeastern USA (Bier et al., 2013, Reynaud et al., 2013).

Vibrio outbreaks commonly occur in tropical and subtropical regions. However, human pathogenic *Vibrio* spp. are becoming increasingly significant in temperate regions (Semenza et al., 2017) with infection cases being associated with seawater warming patterns and heatwave events (Baker-Austin et al., 2013, Sterk et al., 2015). Regular *Vibrio* infection cases occur sporadically also in German coastal waters. While several wound infections caused by *V. vulnificus* were reported exclusively for the Baltic Sea in the recent years (Huehn et al., 2014), various extraintestinal infections (wound infections and otitis) caused by non-O1/non-O139 *V. cholerae* strains were documented to originate from waters of the North Sea (Schirmeister et al., 2014).

Previous environmental studies of potentially pathogenic *Vibrio* spp. in German waters mainly concentrated on the monitoring aspect, trying to shed light on environmental dependencies of their occurrence and abundance patterns and on the detection of virulence-associated genes (Böer et al., 2013, Oberbeckmann et al., 2012, Oberbeckmann et al., 2011a, Hackbusch et al., 2020). The majority of *V. cholerae* and *V. vulnificus* samples distributed throughout the German Bight were found to be of potential clinical relevance due to the presence of multiple virulence factors (Bier et al., 2015, Schirmeister et al., 2014, Hackbusch et al., 2020). It has been suggested that certain virulence factors particularly those of *V. cholerae*, might contribute to its environmental fitness and provide selective advantage (Rahman et al., 2008, Dziejman et al., 2005, Sakib et al., 2018, Hackbusch et al., 2020). However, little is known

about the specific environmental conditions that may favour clinically relevant *Vibrio* strains and support their spread. In the context of ongoing global warming, it has become crucial to investigate the potential of *Vibrio* strains with greater infective risk to survive and thrive in coastal waters.

Pathogen growth potential (PGP) assays are commonly conducted on food or water samples for microbial risk assessment purposes (Farhat et al., 2018, Kim et al., 2012, Vital et al., 2010). PGPs are used to quantify the growth extent of selected pathogenic strains in a sample under defined abiotic conditions. Numerous quantification methods of bacterial growth curves can be applied for the determination of growth parameters in various sample types. These methods underwent a big evolution from the use of plate counts (Gay et al., 1996) and optical density (Mytilinaios et al., 2012) over bioluminescence (Vogel et al., 2014) to the rapid and easy-to-automate total cell counting by fluorescence staining and flow cytometry measurements (Hammes and Egli, 2010, Vital et al., 2010). For a comparison of growth curves between samples or those between strains, variations in population density and growth kinetics are assessed by following growth parameters: Maximum specific growth rate, population density and lag time. Those parameters are commonly estimated by the use of primary growth models (Buchanan et al., 1997, Yoon et al., 2008). Furthermore, secondary growth models enable the analysis of environmental dependencies of those growth parameters (Fernandez-Piquer et al., 2011, Oh et al., 2012).

This study is the first extensive investigation of the pathogen growth potentials of *V. vulnificus* and *V. cholerae* strains in the German Bight. For that purpose, clinically relevant environmental strains possessing multiple virulence-associated genes were grown *in vitro* in a broad range of surface water samples, differing considerably in their physicochemical composition originating from a salinity gradient between the river Elbe and the island Helgoland. Growth kinetics and maximum population densities were monitored in batch assays using a total cell quantification technique by nucleic acid fluorescence staining and flow cytometry measurements. We focused on three main goals in this study: First, we aimed to determine to which extent a spectrum of *in situ* environmental conditions of the German Bight would potentially support the growth of clinically relevant *V. vulnificus* and *V. cholerae* strains. Secondly, we investigated how nutrient limitation alters the growth behaviour of those strains towards salinity and temperature fluctuations by comparing their growth behaviour to that

under high-nutrient conditions. Furthermore, this study focused on analysing the influence of abiotic environmental factors on the growth kinetics of the *V. vulnificus* and *V. cholerae* strains in the German Bight.

Materials & Methods

Bacterial strains

Each one environmental strain of the species *V. vulnificus* (VN – 10012) and *V. cholerae* (VN – 3369) were subject to growth assays. These strains have been isolated from seawater samples of the German Bight, North Sea by health authorities during the German research programs KLIWAS and VibrioNet in 2010 and 2011, respectively. VN-3369 is a vcg C (clinical) *V. vulnificus* strain carrying the virulence-associated genes nanA (sialic acid catabolism) and manIIA (mannitol fermentation) isolated by Niedersächsisches Gesundheitsamt (Lower Saxony State Health Office, NLGA). VN-10012 is a *V. cholerae* strain carrying following virulence-associated genes: VSP-II (7^{th} pandemic island II), *chxA* (Cholix Toxin), *hlyAET* (El Tor variant hemolysin) and *rtxC* (Repeats-in-toxin) isolated by the Lower Saxonian State Office for Consumer Protection and Food Safety (LAVES).

Strain reactivation and pre-culture

Prior to growth assays, species identification of the *Vibrio* strains was conducted by Matrix-assisted-laser-desorption-ionization Time-of-flight Mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Germany) described by Erler et al. (2015). To create high quality mass spectra, a previously described formic acid/acetonitrile protein extraction method was conducted on biomass of single colonies (Mellmann et al., 2008). After successful identification (MALDI-TOF Score: 3.5), the strains were transferred into fresh Alkaline Peptone Water (APW, pH 8.5) and incubated over night at 37 °C. This fresh culture was used as a pre-culture for subsequent growth assays.

Before inoculation of the growth assays, bacterial cells of the pre-cultures were washed three times in 1x PBS buffer (Phosphate buffered saline, Thermo Fisher Scientific, USA) by centrifugation (5 min at 6000 rpm). The resulting pellet was resuspended in fresh 1x PBS buffer for further use.

High-nutrient growth media preparation

Marine broth (Peptone, yeast extract, FePO4, pH 7.6) in salinities ranging from 0.7 to 38.4 (n = 19) and prepared with sterile seawater from the Helgoland Reede (North Sea, Germany, 54° 11.3’ N, 7° 54.0’ E) was used as high-nutrient growth media (Supplementary material, Table 1). All growth media were autoclaved, filtered through 0.22 µm sterile membrane filters (ACN, Sartorius Stedim biotech, USA), and stored at 4 °C until further use.

Seawater sample preparation

Surface water samples of monthly research cruises (May to October 2015 and May 2016 to January 2017) in a salinity gradient of the German Bight extending from the Elbe estuary to the island Helgoland were used as low-nutrient growth media for Pathogen Growth Potential (PGP) assays. Water samples were collected in a previous study with a rosette water sampler equipped with a CTD Probe (CTD90, Sea and Sun Technology, Germany) at a depth of approximately 1 m (Hackbusch et al., 2020). 1.5 l seawater of each sampling point was filtered through a 0.45 µm sterile membrane filter (ACN, Sartorius Stedim biotech, USA) and immediately stored in 0.5 l Nalgene bottles at -20 °C until further processing. Following the protocol of (Vital et al., 2010), water samples were pasteurized (60 °C, 30 min) in a water bath (63 °C) and sterile filtered through 0.2 µm GTTP filters (Merck Millipore, USA) immediately before usage.

Spectrophotometric high-nutrient growth assays

High-nutrient growth was monitored spectrophotometrically [OD_{600}] in batch assays in Marine broth with different salinities (0.7 – 38.4) at temperatures between 10 and 26 °C (n = 9) using the temperature-controlled microplate reader Infinite M200 (Tecan Group Ltd., Switzerland).

Prior to these growth assays, all growth media (n = 19; Supplementary material, table 1) were adjusted overnight to reach the respective target temperatures in the medium before inoculation. The acceptable incubation temperature range was set to +/- 0.5 °C of the target temperature and was monitored throughout the incubation time. To prevent excessive

sample evaporation during the incubation, a tray filled with water was placed into the incubation chamber.

Growth assays were conducted in triplicates in each growth medium (n = 19) using sterile flat bottom 96-well plates (Corning Inc., USA) with sterile lids. Each growth medium was inoculated with the pre-culture with an initial cell concentration of 0.002 OD_{600} and incubation started immediately. Absorbance (600 nm) was measured automatically every 15 minutes until the stationary phase in each growth medium was reached. To prevent sedimentation, the 96-well plates were subjected to shaking for 2 min in an orbital amplitude of 5 mm prior to each measurement. Blanks were included in duplicates for each growth medium and the blanks were subtracted from each respective sample for all time points. In case of condensation on the lid at low incubation temperatures (< 14 °C), absorbance data were excluded for the respective time until no visible condensation was detected.

Flow cytometric Pathogen Growth Potential assays (PGP)

Pathogen Growth Potentials (PGP) of the clinically relevant *Vibrio* strains were analysed in seawater samples of the German Bight at *in situ* temperature using the flow cytometer Accuri™ C6 (BD Biosciences, Belgium) with an accessory multi-well autoloader (BD CSampler™; BD Biosciences, Belgium).

Batch growth assays (PGP) at their respective *in situ* temperatures in categories of 1 °C steps (8.5 – 20.5 °C) were conducted for each bacterial strain, comprising of a maximum of eight water samples per batch assays. Each pasteurized and filtered seawater sample was aliquoted (35 mL) into 50 mL-vented bioreactor vials (Corning Inc., USA) in triplicates. Prior to the PGP assays, all seawater samples were adjusted overnight to reach the respective target temperatures in the seawater before inoculation.

Each *Vibrio* strain was pre-cultivated as described above and separately inoculated into each sterile seawater sample vial with a starting concentration of approximately 500 cells ml^{-1}. One extra vial of each seawater sample served as a negative control to assess for possible contamination. PGP incubation started immediately in temperature-controlled incubation chambers under rotation (20 rpm) with a maximum variation of +/- 0.5 °C of the set *in situ*

temperature. Growth in each seawater sample was monitored until the stationary phase was reached, but for a maximum of six days. For that, a 166-µl aliquot of each vial was aseptically transferred into a sterile round-bottom 96-well plate (Sarstedt AG & Co. KG, Germany) in triplicates twice a day and cells were fixed with formaldehyde (1.5 % final concentration) for 45 min at room temperature. Fixed cells were fluorescently stained with SYBR Green I (8X final concentration) for 20 min at room temperature in the dark. Flow cytometry was used for cell quantification [N ml^{-1}] for 90 s at a speed of 35 µl min^{-1} with the threshold set on green fluorescence (FL1-H; 1000). A flush with MilliQ water followed after each sample batch (3 x 3 replicates) to prevent s. For the homogenization of the samples prior to each measurement and to prevent sedimentation, 96-well plates were agitated for 15 sec prior to each measurement. Events were gated on green/red fluorescence (FL1-H/FL3-H) density plots for the determination of total cell concentrations per sample. Finally, blanks were subtracted from the cell counts of each sampling point. In case of a contamination of a blank, all samples of this seawater sample were discarded and the assay was repeated.

Growth modelling

Primary growth modelling was conducted for computing the growth parameters population density (C), maximum specific growth rate (SGR) and lag time (LT). Growth curves, including all replicates per sample (9 for PGP assays, 3 for high-nutrient assays) were fitted using the modified Gompertz equation (1) according to Zwietering et al. (1990). The equation was used as follows:

$$Y = N_0 + C \times e^{-e^{\frac{2.718 \times SGR}{C} \times (LT - t) + 1)}} \qquad (1)$$

where Y is the cell concentration (OD_{600} or log N ml^{-1}), N_0 is the initial cell concentration, C is the difference between the initial and final cell concentration, SGR is the maximum specific growth rate (log N h^{-1}), LT is the lag time (h) and t is time (h). Constrains of LT ≥ 0 were defined in case otherwise no fitting was possible.

Secondary modelling was conducted to analyse the temperature dependency of SGR using the Square-root equation (Ratkowsky et al., 1982) and of LT using the linear Arrhenius equation (Davey 1989). The Square-root equation for SGR was used as follows:

$$SGR = b \times (T - T_{min})^2 \quad (2)$$

where b is the regression coefficient, T is the *in situ* temperature and T_{min} is the theoretical minimum temperature required for the growth of the organism to occur.

The linear Arrhenius equation for LT (Davey, 1989) was used as follows:

$$LT = a + \frac{b}{T} + \frac{c}{T^2} \quad (3)$$

where a, b and c are regression coefficients without biological meaning T is the *in situ* temperature.

The software GraphPad Prism 6.0 (GraphPad Software, USA) was used for all primary and secondary modelling. The goodness-of-fit of the secondary models was evaluated by the coefficient of determination (R^2).

Statistical analysis

Spearman Rank Order Correlation was used to analyse linear correlations among growth parameters and between growth parameters and physico-chemical parameters with a significance level of $p < 0.05$. Non-parametric Mann Whitney U rank sum tests were conducted for the detection of significant differences among the species *V. vulnificus* and *V. cholerae* regarding growth parameters. All statistical analysis was carried out using the software SigmaPlot 12 (Systat Software Inc., USA).

Environmental parameters (temperature, salinity, SiO_4, PO_4, NO_2, NO_3, NH_4) of PGP samples (Hackbusch et al., 2020) were log (x+1)-transformed and normalized prior to statistical analysis. Principal Component Analysis (PCA) was conducted on all physico-chemical parameters using the software PRIMER 6 (PRIMER-E Ltd, New Zealand). To examine the relationship between each PGP growth parameters and concomitant physico-chemical parameters, forward stepwise multiple linear regression analyses were conducted using PCA scores of the first three PCA axes. To overcome the effect of multicollinearity, PCA axes replaced the original environmental data as explanatory variables, referred as latent variables. The approach of principal component regression (PCR) (e.g. Hotelling (1957), Lucas et al.

(2016)) removes multicollinearity completely, since PCA axes are perfectly uncorrelated (orthogonal) to each other.

Results

V. vulnificus and *V. cholerae* growth under high-nutrient conditions

Growth curves of *V. vulnificus* could be fitted in 83 % (142) of the assays with primary modelling (Gompertz equation) (Table 1). Minimum temperature required for the growth of *V. vulnificus* was 12 °C. Extreme salinities (36 - 38 and 0.7 - 3) limited growth at 12 °C, while the lowest salinity (0.7) additionally limited growth at most temperatures (12 - 20 & 26 °C) (Supplementary material, Figure 1). These samples could not be fitted with the primary growth model. No case of population decline was observed under high-nutrient conditions (Table 1). Population density (C) of *V. vulnificus* ranged from 0.05 to 0.73 OD (mean = 0.28), the maximum specific growth rate (SGR) ranged from 0.0008 to 0.06 OD h^{-1} (mean = 0.02) and the lag time (LT) ranged from 0 to 156 h (mean = 30.8). Spearman rank analysis of *V. vulnificus* growth parameters revealed a significant negative correlation between SGR and LT (rs = -0.87, $p < 0.001$). Linear correlations with physico-chemical parameters were detected between SGR and temperature (rs = 0.85, $p < 0.001$), LT and temperature (rs = -0.89, $p < 0.001$) as well as between C and salinity (rs = -0.7, $p < 0.001$).

Growth curves of *V. cholerae* could be fitted in 98 % (167) of the assays with primary modelling (Gompertz equation) (Table 1). *V. cholerae* growth was detected at all temperatures (10 – 26 °C). At low salinities (0.7 - 5) and lowest temperatures (10 & 12 °C), growth of *V. cholerae* was limited (Supplementary material, Figure 1). These samples could not be fitted with primary modelling. No case of population decline was recorded at any condition. C of *V. cholerae* ranged from 0.1 to 0.94 OD (mean = 0.4), SGR ranged from 0.002 to 0.07 OD * h^{-1} (mean = 0.02) and LT ranged from 2.1 to 153 h (mean = 29.5). C was high at salinities below 20 for the entire temperature range (Figure 1). Spearman rank analysis of *V. cholerae* growth parameters revealed a significant positive correlation between SGR and C (rs = 0.66, $p < 0.001$) and a significant negative correlation between SGR and LT (rs = -0.71, $p < 0.001$). Linear correlations with physico-chemical parameters were detected between SGR and temperature (rs = 0.65, $p < 0.001$), LT and temperature (rs = -0.98, $p < 0.001$), C and salinity (rs = -0.83, $p < 0.001$), SGR and salinity (rs = -0.65, $p < 0.001$) as well as between LT and salinity (rs = 0.19, $p < 0.05$).

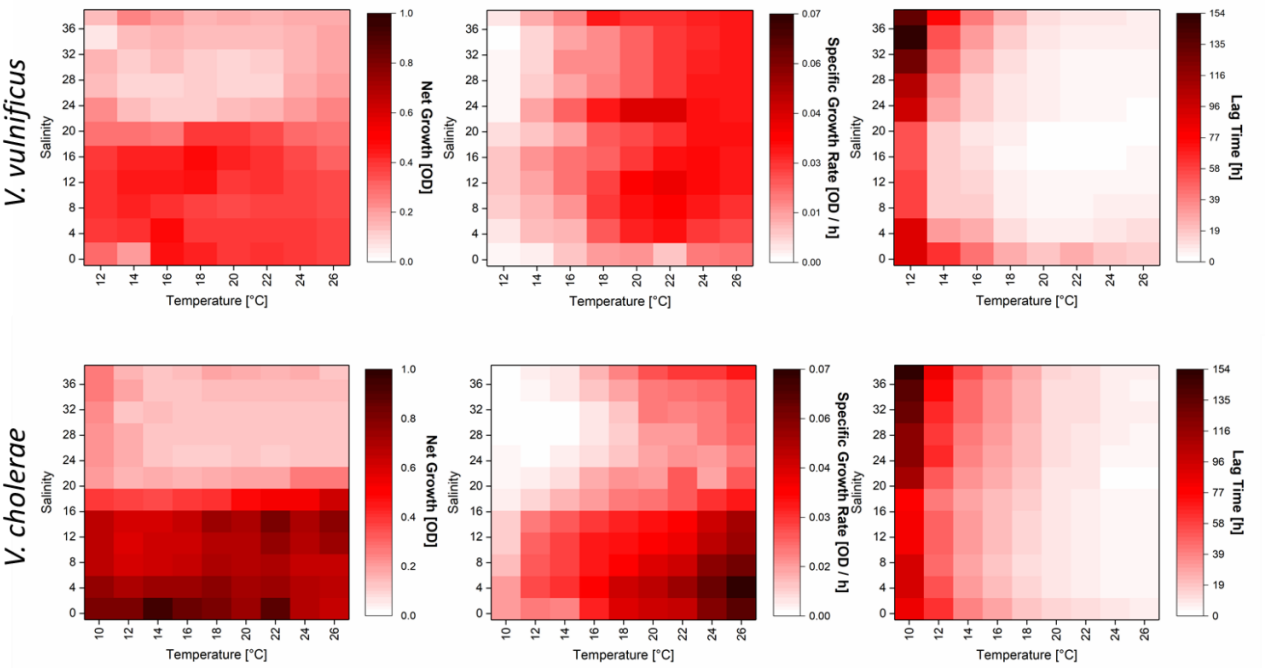

Figure 1: *V. cholerae* and *V. vulnificus* growth under high-nutrient conditions changing with temperature and salinity. Growth parameters population density (net growth, C), maximum specific growth rate (SGR) and lag time (LT) are depicted in the colour shadings. Smoothing was done by kriging

Comparing the growth parameters between *V. vulnificus* and *V. cholerae*, significant differences in C ($U = 8829$, $p < 0.001$) and LT ($U = 11254$, $p < 0.001$) were observed using the nonparametric Mann Whitney Rank Sum Test. SGR of both species could be linearly fitted with temperature (*V. vulnificus:* $SGR = 0.003 * T - 0.028$; $R^2 = 0.76$; *V. cholerae:* $SGR = 0.002 * T - 0.013$; $R^2 = 0.56$) (Figure 3). Population density and lag time on the other hand, showed non-linear patterns for both species: C with salinity and LT with temperature.

Pathogen growth potentials (PGP) of *V. vulnificus* and *V. cholerae* in surface waters of the German Bight

69 % (49) of all *V. vulnificus* growth curves could be successfully fitted with primary modelling (Gompertz equation) (Table 1). Among these, 19 % (9) showed a population decline (SGR < 0) at 8.5 to 14.5 °C and at salinities between 17.8 and 32.9. Growth (SGR > 0) was detected in 56 % (40) of the samples between 12.5 and 20.5 °C and at salinities between 16.7 and 33.4. 17 %

of the samples (12) could not be converged with the primary growth model, among these, 4 samples showed population decline at 10.5 – 15.5 °C and at salinities between 29 and 32.9. Population density of *V. vulnificus* in PGP assays ranged from 0.4 to 10^3 N ml^{-1} (mean = 40 N ml^{-1} h^{-1}), SGR ranged from 1 to 1.6 N ml^{-1} h^{-1} (mean = 1.1 N ml^{-1} h^{-1}) and LT ranged from 12 to 164 h (mean = 56 h). Population density of *V. cholerae* increased with increasing temperature but displayed a sudden drop at 18.5 °C to values between 16 – 40 N ml^{-1}. SGR showed an increase with increasing temperatures, starting from 12.5 °C, while lag times decreased with increasing temperatures. Above 17.5 °C, *V. vulnificus* revealed a sudden drop in LT values to 12 to 53 h (Figure 2).

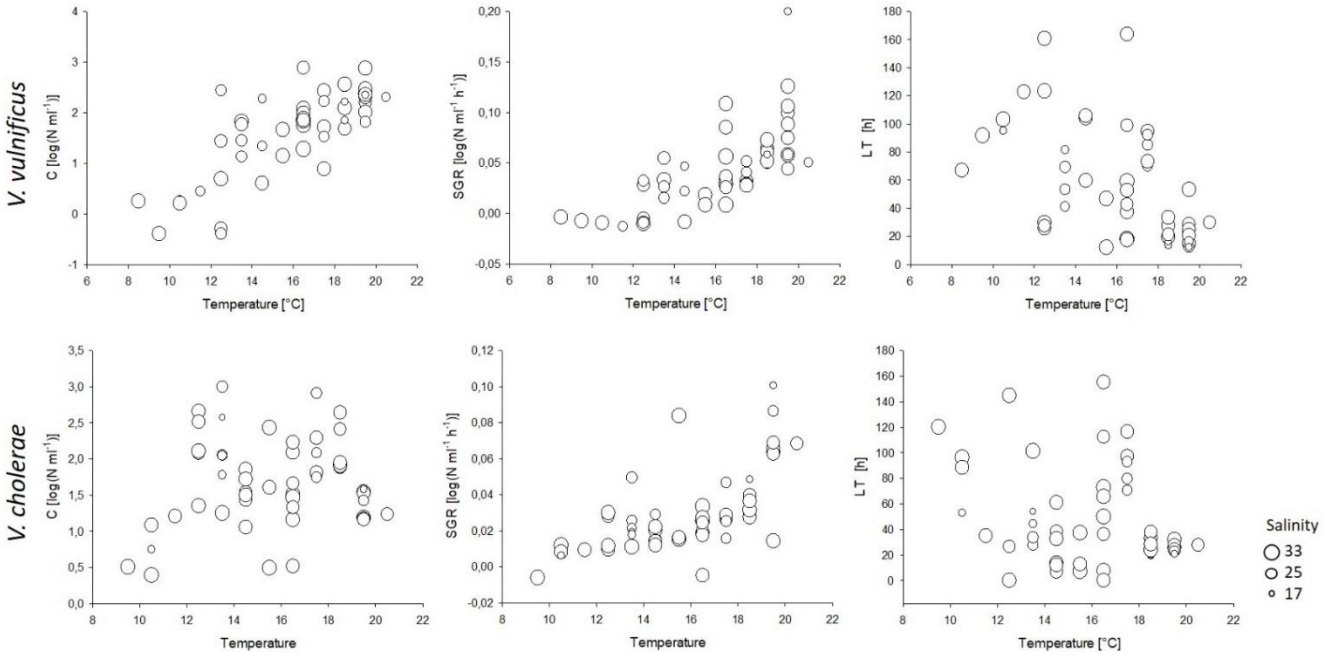

Figure 2: *V. cholerae* and *V. vulnificus* PGP growth parameters changing with temperature. Prevailing salinity is depicted in the bubble size.

Spearman rank analysis of *V. vulnificus* growth parameters revealed a significant positive correlation between SGR and C (rs = 0.77, $p < 0.001$), negative correlations between SGR and LT (rs = -0.65, $p < 0.001$) and between C and LT (rs = -0.43, $p < 0.01$). Linear correlations with physico-chemical parameters were detected between all growth parameters and temperature (C: rs = 0.71, $p < 0.001$; SGR: rs = 0.8, $p < 0.001$; LT: rs = -0.56, $p < 0.001$); and between SGR

and salinity (*rs* = -0.32, $p < 0.05$). Population density of *V. vulnificus* showed a linear regression with temperature (C = 0.21 * T -1.71; $R^2 = 0.59$) (Figure 3).

80 % (57) of all *V. cholerae* growth curves could be successfully fitted, among which two samples showed population decline (SGR < 0) at 9.5, at 16.5 °C and at salinities of 32.9 and 30.7, respectively. Growth was detected (SGR > 0) in 55 samples at 10.5 to 20.5 °C with salinities between 14.5 and 33.3. Seven percent of the samples (5) showing population decline at 8.5 to 16.5 °C and salinities of 29 – 33.4 could not be converged with the primary growth model. Population density of *V. cholerae* in PGP assays ranged from 2.5 to 10^3 N ml^{-1} (mean = 50 N ml^{-1}), SGR ranged from 1 to 1.3 N ml^{-1} h^{-1} (mean = 1.1 N ml^{-1} h^{-1}) and LT ranged from 0 to 155 h (mean = 44 h). SGR showed an increase with increasing temperatures, especially above 18.5 °C (Figure 2). Population density had no clear temperature or salinity pattern but dropped above 18.5 °C while LT decreased with increasing temperatures. Above 17.5 °C, *V. cholerae* revealed a sudden drop in LT values to 20 to 38 h (Figure 2).

Spearman rank analysis of *V. cholerae* growth parameters revealed a significant negative correlation between C and LT (*rs* = -0.38, $p < 0.01$). Linear correlations with physico-chemical parameters were detected between SGR and temperature (*rs* = 0.73, $p < 0.001$) and between SGR and salinity (*rs* = -0.29, $p < 0.5$). Comparing the growth parameters between both species under low-nutrient conditions, no significant differences were detected using the non-parametric Mann Whitney Rank Sum Test.

Table 1: Primary modelling of *V. vulnificus* and *V. cholerae* growth in high-nutrient (Broth) and low-nutrient (PGP) conditions. Growth curves are shown in the categories "Fitted", which could be fitted using the primary growth model (modified Gompertz equation) and "Not fitted", which could not be fitted with this model although constrains of LT > 0 were used. For the fitted growth curves the category "Growth" is defined as SGR > 0 and "Decline" as SGR < 0. Non-fitted growth curves were categorized into "No growth" when cell numbers remained unchained or into "Decline" when cell numbers decreased compared to the initial cell concentration. Growth data which could not be converged despite an increase in cell concentrations were excluded from this table (10 for *V. vulnificus* and 9 for *V. cholerae*).

			V. vulnificus		***V. cholerae***	
			N	**Temperature (salinity)**	**N**	**Temperature (salinity)**
Broth (total 171)	**Fitted**	**Growth**	142 (83 %)	12 °C (4.8 - 34.2) - 26 °C (2.6 - 38.4)	167 (98 %)	10 °C (6.8 - 38.4) - 26 °C (0.7 - 38.4)
		Decline	-	-	-	-
	Not fitted	**No growth**	29 (17 %)	10 °C (all); 12 °C (36 - 38); all T (0.7)	4 (2 %)	10 °C (0.7 - 4.8); 12 °C (0.7)
		Decline	-	-	-	-
PGP (total 71)	**Fitted**	**Growth**	40 (56 %)	12.5 - 20.5 °C (16.7 - 33.4)	55 (77 %)	10.5 - 20.5 °C (14.5 - 33.3)
		Decline	9 (13 %)	8.5 - 14.5 °C (17.8 - 32.9)	2 (3 %)	9.5 °C (32.9); 16.5 °C (30.7)
	Not fitted	**No growth**	8 (11 %)	8.5 - 16.5 °C (14.2 - 33.3)	-	-
		Decline	4 (6 %)	10.5 - 15.5 °C (29 - 32.9)	5 (7 %)	8.5 - 16.5 °C (29 - 33.4)

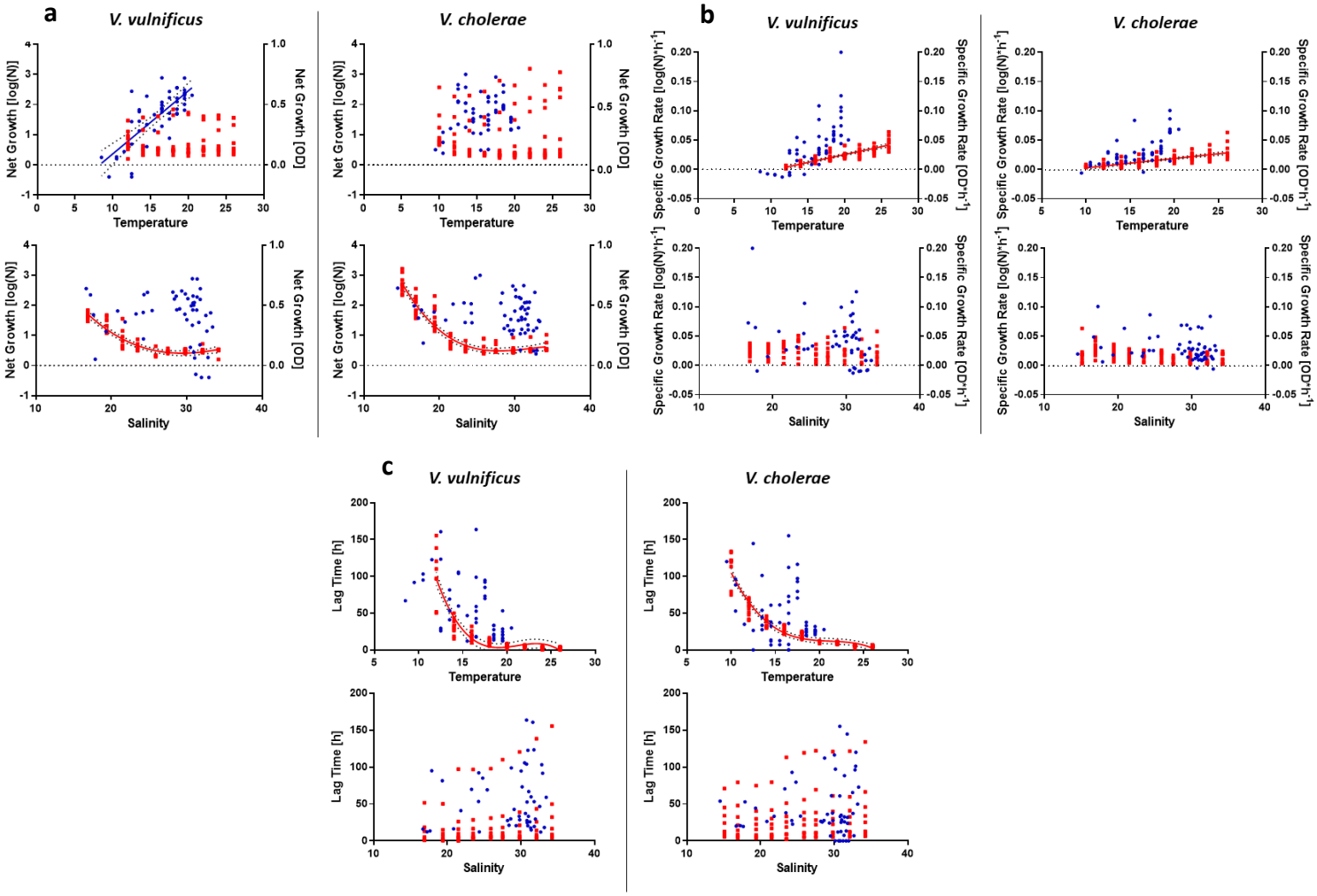

Figure 3: Changing growth parameters of *V. vulnificus* and *V. cholerae* under low-nutrient (blue circles) and high-nutrient (red squares) conditions at a temperature and salinity range of the German Bight. Depicted are a) Population density (net growth), b) maximum specific growth rate and c) lag times.

Influencing factors of *V. vulnificus* and *V. cholerae* growth kinetics in the German Bight

The temperature dependency of SGR of both species could be modelled with the secondary square-root model (Ratkowsky equation). For *V. vulnificus* this model showed an R^2 of 0.5 and a slope of 0.027 (Figure 4). The square-root model of *V. cholerae* had an R^2 of 0.46 and a slope of 0.018. The minimum growth temperature of *V. vulnificus* and *V. cholerae* revealed by the model were 9.1 °C and 6.6 °C, respectively.

Secondary modelling for the analysis of the temperature dependency of LT (Davey equation) resulted in low R^2 values, with 0.3 for *V. vulnificus* and 0.1 for *V. cholerae* (Figure 5).

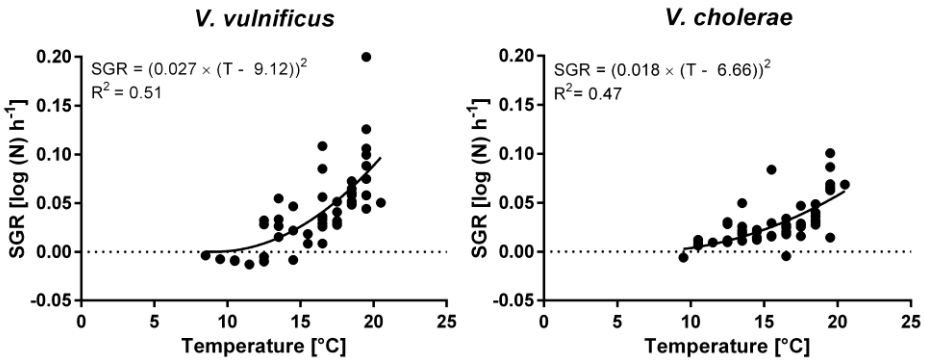

Figure 4: Secondary models of *V. cholerae* and *V. vulnificus* maximum specific growth rates under low-nutrient conditions (PGP). Temperature dependency of the maximum specific growth rate was modelled using a square-root equation.

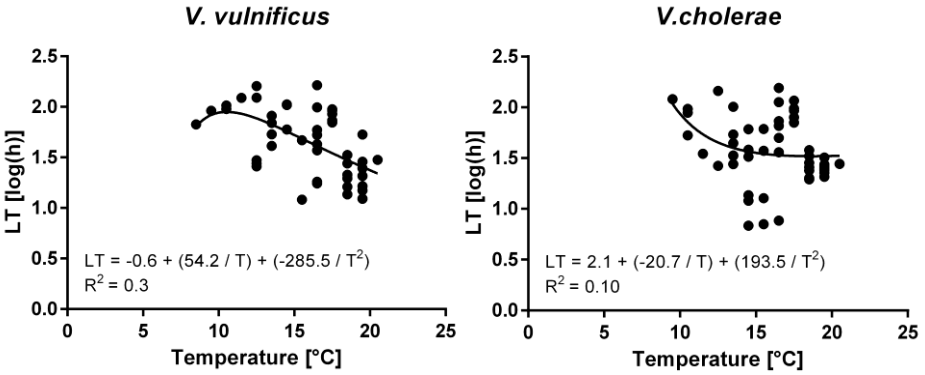

Figure 5: Secondary models of *V. cholerae* and *V. vulnificus* lag times under low-nutrient conditions (PGP). Temperature dependency of the lag time was modelled using the linear-Arrhenius equation.

Principal component analysis (PCA) of physico-chemical parameters resulted in three main principal components with PC1 explaining 61.7 %, PC2 16.5 % and PC3 explaining 10.9 % of the total variation (Figure 6). PC1 is mainly represented by salinity and nutrients (silicate, phosphate, nitrate, nitrite, ammonium). PC2 is mainly represented by temperature, while PC3 is mainly represented by nitrate and ammonium (Figure 6).

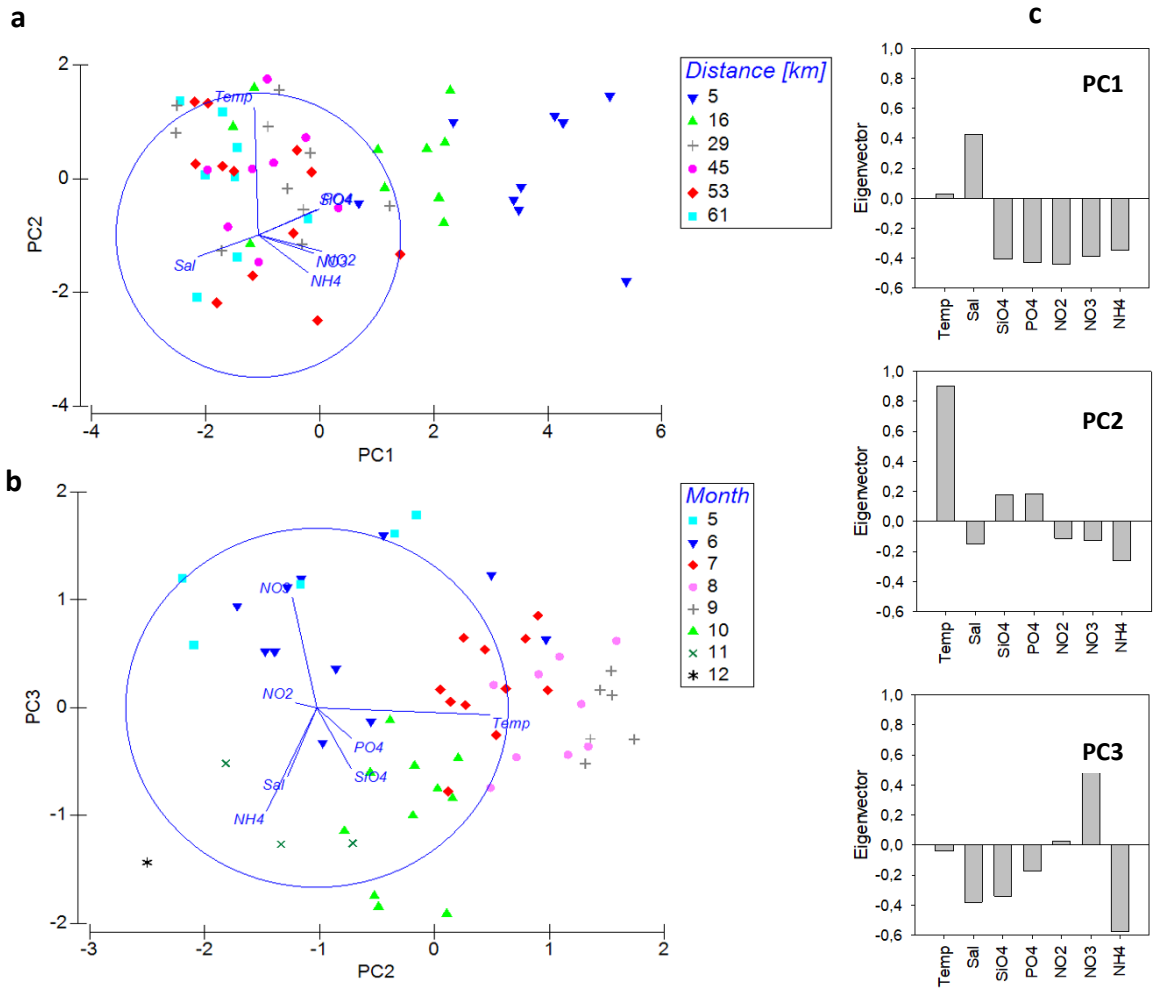

Figure 6: Principal Component Analysis (PCA) of prevailing physico-chemical conditions of seawater samples used in PGP assays. The first three PC axes are shown in the biplots (a and b). Eigenvector values of each PC axis is given in bar charts for each physico-chemical parameter (c).

All *V. vulnificus* PGP growth parameters could significantly be explained by PC2 (p (model) < 0.001) via Principal component regression (Table 2). *V. cholerae* growth parameters in PGP assays revealed a variable dependency, in which C could significantly be explained by PC3 (R^2_{adj} = 0.59; p (model) = 0.04) (Table 3). SGR on the other hand could significantly be explained by PC2 (R^2_{adj} = 0.44; p (model) < 0.001), whereas no principal component could significantly explain LT.

Table 2: Principal component regression of *V. vulnificus* PGP growth parameters with the first three PC axes.

Growth parameter	p (model)	R^2 (adj)	PC included in model	Coefficient	p (var)	F-to remove (var)
C	**<0.001**	**0.59**	Constant	1.63		
			PC2	**0.60**	**<0.001**	**67.13**
SGR	**<0.001**	**0.50**	Constant	0.04		
			PC2	**0.03**	**<0.001**	**48.72**
LT	**<0.001**	**0.29**	Constant	55.61		
			PC2	**-20.29**	**<0.001**	**20.65**

Table 3: Principal component regression of *V. cholerae* PGP growth parameters with the first three PC axes.

Growth parameter	p (model)	R^2 (adj)	PC included in model	Coefficient	p (var)	F-to remove (var)
C	**0.04**	**0.59**	Constant	1.69		
			PC3	**0.19**	**0.04**	**4.37**
SGR	**<0.001**	**0.44**	Constant	0.03		
			PC2	**0.01**	**<0.001**	**44.07**
LT	-	-	-	-	-	-

Discussion

Vibrio vulnificus and *Vibrio cholerae* are commonly known as important human pathogens associated with gastroenteritis and wound infections (Thompson et al., 2004a). Several studies have shown that both species, *V. vulnificus* and *V. cholerae*, are widely present in the temperate waters of the German Bight, North Sea (Böer et al., 2013, Hackbusch et al., 2020). Most of the isolated strains and community samples from this region are shown to carry multiple virulence-associated genes, representing a potential clinical relevance (Bier et al., 2015, Schwartz et al., 2019, Hackbusch et al., 2020) but the environmental control of their growth and persistence are poorly understood in the aquatic environment. To our knowledge, this study is the first to provide a comprehensive growth potential analysis of environmental *V. vulnificus* and *V. cholerae* strains carrying multiple virulence-associated genes, targeting a broad range of abiotic conditions prevailing in a salinity gradient of the German Bight.

Clinically relevant *V. vulnificus* and *V. cholerae* are able to spread in the German Bight

Temperature and salinity are prominent factors in *Vibrio* spp. ecology and population dynamics (Kaspar and Tamplin, 1993, Randa et al., 2004). In this study, *V. vulnificus* (vcg type C *, nanA*$^{+}$*, manIIA*$^{+}$) and *V. cholerae* (VSP-II^{+}*, chxA*$^{+}$*, hlyAET*$^{+}$*, rtxC*$^{+}$) strains were able to grow in most of the seawater samples (PGP) which represented a wide range of salinity-temperature conditions prevailing in the German Bight (temperatures between 10.5 to 20.5 °C and salinities between 14.5 – 33.4). Both species showed no clear growth pattern as a response to changing salinities (Figure 3, Table 2 & 3, Supplementary Figure S2.1). Environmental *V. vulnificus* and *V. cholerae* prefer brackish waters with reports of occasional occurrences at higher salinities (Böer et al., 2013) especially increasing in abundances when high temperatures prevail in coastal environments (Randa et al., 2004, Hackbusch et al., 2020).

In this study, the minimum temperature required for *V. vulnificus in vitro* growth in seawater was 12.5 °C while *V. cholerae* could even grow at 10.5 °C. Based on the secondary modelling of the specific growth rate with temperature, the theoretical minimum temperature needed for growth for *V. vulnificus* was 9.1 °C and for *V. cholerae* 6.5 °C, both lower than the respective minimum temperatures for *in vitro* growth observed in this study. In the German Bight, it has

been demonstrated that *V. vulnificus* occurred *in situ* only above 13.3 °C while the *V. cholerae* population was still detectable at 8.5 °C (Hackbusch et al., 2020). In accordance with Materna et al. (2012), we found indications that the realized ecological range of *V. vulnificus* and *V. cholerae* are a limited section of their fundamental niches. With increasing temperatures, *V. vulnificus* showed a successive increase of population densities and specific growth rates together with a decrease in lag times. Concurrent with these findings, our previous monitoring study in the German Bight revealed a high temperature dependency of *V. vulnificus* with a rapid elevation of abundances with increasing temperatures (Hackbusch et al., 2020). Additionally, a rapid growth response (LT: 12 – 53 h) at temperatures above 17.5 °C was observed in this study. Our results confirm recent findings of Semenza et al. (2017) that the risk for *V. vulnificus* infections in European countries increases successively above Sea Surface Temperature (SST) of 16°C.

V. cholerae displayed similar growth patterns to *V. vulnificus* as growth kinetics accelerated (increase of SGR, decrease of LT) with increasing temperature. However, contrary to *V. vulnificus*, the population density of *V. cholerae* showed no clear temperature or salinity response. However, a rapid growth response to high temperatures was detected also for *V. cholerae* above 17.5 °C (LT: 20 – 38 h), as seen with *V. vulnificus*. Overall, the results of this study highlight the high potential of clinically relevant *V. vulnificus* and *V. cholerae* to grow in the German Bight under ambient conditions. The ability for a rapid response to warm temperatures might result in frequent *V. vulnificus* and *V. cholerae* blooms in the German Bight, probably leading to an increase in infection cases.

Nutrient limitation changes the growth behaviour of clinically relevant *V. vulnificus* and *V. cholerae* strains towards salinity and temperature fluctuations

Besides physicochemical factors such as temperature and salinity, nutrient availability (mainly carbon) is known to drive the growth potential of pathogens (Prest et al., 2016) by governing cellular growth kinetics and population sizes. In coastal environments, *Vibrio* spp. abundances have been reported to be nutrient dependent (Hackbusch et al., 2020, Böer et al., 2013). Comparing the growth patterns between low-nutrient (seawater) and high-nutrient (broth) conditions at similar temperature and salinity ranges, this study showed that nutrient

availability significantly changed the growth response of *V. vulnificus* and *V. cholerae* to temperature and salinity fluctuations (Table 1).

The biggest differences in temperature and salinity dependence between low- and high-nutrient conditions was detected in population densities. Under high-nutrient conditions, the population density of *V. vulnificus* and *V. cholerae* was highly influenced by salinity, while temperature seemed to have no detectable effect (Figure 3). Under low-nutrient conditions on the contrary, the population density was not influenced by salinity (Supplementary Material, Figure 2.2). *V. vulnificus* showed a linear increase of population density with increasing temperature while no pattern was observed for *V. cholerae* (Figure 2). Generally, the ability of both species to grow decreases with increasing salinity levels, given their reported adaptation to estuarine conditions, which is particularly the case for *V. cholerae*. The population density was limited by high salinities (> 20) (Supplementary material, Figure 1) under high-nutrient conditions in this study, both species' ability to reach high population densities at elevated salinities under low-nutrient conditions. In line with these findings, the clinically relevant *Vibrio* spp. previously isolated from the German Bight, an estuarine ecosystem with pronounced temporal and spatial fluctuations in salinity and nutrient concentrations, seem to be well adapted towards those environmental challenges.

Irrespective of nutrient availability, the maximum specific growth rate of both *Vibrio* species showed temperature dependence without revealing any salinity pattern. Interestingly, the effect of temperature on SGR was elevated under low-nutrient conditions (square-root) compared to high-nutrient conditions (linear) due to a successively increasing slope with increasing temperatures. *Vibrio* spp. are capable of very rapid growth in laboratory media (Ulitzur, 1974), but have been reported to reach high growth rates also in the environment. Usually, the *Vibrio* taxa belong to rare species (0 - 2 %) of marine microbial communities but environmental time-series analysis showed that they are capable of shortly becoming predominant (54 %) community members by forming blooms in the water column (Gilbert et al., 2012).

Under high-nutrient conditions, both species showed a pronounced temperature pattern and displayed very short lag times at high temperatures (above 17.5 °C). Interestingly, low temperature-high salinity conditions (semi-optimal conditions) prolonged the lag times of both species irrespective of nutrient availability.

Apart from growth requirements, with this study we could show that nutrient limitation can promote the decline of *V. vulnificus* and *V. cholerae* populations, due to decreasing cell concentrations and negative specific growth rates. These cases of population decline (3 for *V. cholerae*, 9 for *V. vulnificus*) occurred at high salinity (29 - 33.4) and low temperature (8.5 – 16.5 °C) conditions. Opposed to that, no case of population decline occurred under high-nutrient conditions with comparable salinity and temperature ranges. One possible explanation for these findings is the entrance into the viable-but-not-culturable (VBNC) state of these species as a survival strategy to endure low temperatures and starvation (Wong and Wang, 2004, Rao et al., 2014). In the VBNC state morphological changes occur from rod to coccoid cell shapes (Fernández-Delgado et al., 2015), probably leading to a decreased detection of those cells by flow cytometry.

Comparing the growth requirements of both species, *V. vulnificus* and *V. cholerae*, *V. vulnificus* growth appears more sensitive to changes in temperature than *V. cholerae*, especially for population density and specific growth rates. The minimum temperature required for growth in high-nutrient broth and in low-nutrient seawater is similar for each species with 12 and 12.5 °C for *V. vulnificus*, respectively, and 10 and 9.5 °C for *V. cholerae* (10 °C was the minimum target temperature in broth). This indicates, that for both species, nutrients do not influence the minimum temperature required to grow.

Influencing factors of *V. vulnificus* and *V. cholerae* growth kinetics in the German Bight

Physico-chemical parameters influencing the growth behaviour of *V. vulnificus* and *V. cholerae* under low-nutrient conditions in seawater samples were identified by principal component regression (PCR) analyses. Additionally, secondary growth models were applied for the non-linear analysis of the temperature effect on specific growth rates and lag times.

From our results the growth potential *of V. vulnificus* in seawater samples is highly temperature dependent, as population density (C) and growth kinetics parameters (SGR and LT) could be explained exclusively by temperature, as depicted by PCR and secondary modelling results (Figure 4, Table 2). For *V. cholerae* on the contrary, only the specific growth rate (SGR) could be explained by temperature, as PCR analysis and secondary modelling revealed (Figure 4, Table 3). Generally, *V. vulnificus* responded with a strong acceleration of

growth kinetics (higher SGR, lower LT) the higher temperatures prevailed, given the slopes of the secondary growth models (Figure 4 & 5). This phenomenon was less pronounced but still prevailing with *V. cholerae*. Climate change is assumed to favour the geographic distribution of the pathogenic strains and will likely lead to more frequent *Vibrio* infection incidences (Martinez-Urtaza et al., 2010, Baker-Austin et al., 2013). This theory is further reinforced by our study, as increasing temperatures will probably lead to a growing potential of clinically relevant *V. vulnificus* and *V. cholerae* blooms in the German Bight. No salinity dependent pattern of growth kinetics was detected for the broad range of salinities covered by this *in vitro* study.

The population density of *V. cholerae* did not show any temperature or salinity driven pattern but was rather explained by the combination of NO_3 and NH_4 (PCR; Table 3). Recently, similar results were reported, with a prevalent dependence of *V. cholerae* abundances in the German Bight on nitrite (NO_2), which in turn strongly correlates with NO_3, NH_4, PO_4 and cDOM (Hackbusch et al., 2020). The German Bight experiences sudden nitrogen input through freshwater discharge events. With ongoing climate change, extreme events are expected in more frequently future, as heavy rainfall and subsequent heavy river discharge events (van Vliet et al., 2013). Based on the results of this study, clinically relevant *V. cholerae* abundances can be expected to increase in the German Bight estuary.

In addition, the lag time of *V. cholerae* could not be modelled properly, neither by using secondary models for the non-linear effect of temperature, nor by Principal Component Regression for the effect of concomitant abiotic factors. The lag time represents the adaptation time to prevailing environmental conditions including numerous molecular transformations such as synthesis of molecular components, repair of molecular damage and preparing for exponential growth (Rolfe et al., 2012). Lag time of the clinically relevant *V. cholerae* strain seems to depend on other factors, which were not included in this study. One of those missing factors might be surface attachment, of which *V. cholerae* readily makes use of as a response to nutrient limitation. However, our results still underscore previous *V. cholerae* abundance monitoring findings in the German Bight, where this species was detected only sporadically in low concentrations at coastal sites and was rather unpredictable in their occurrence (Hackbusch et al., 2020, Böer et al., 2013). Overall, we could show that the growth behaviour of *V. cholerae* opposed to *V. vulnificus*, is less predictable in the German Bight by

solely abundance analyses. Our findings highlight that the growth behaviour of *V. cholerae* might be attributed to strong physicochemical gradients typically found in estuarine regions, highly limiting its spreading while its adaptation time to environmental conditions seems to depend on other factors, presumably attachment or biotic factors.

Conclusion

The findings in this paper demonstrated that frequent blooms of clinically relevant *V. vulnificus* and *V. cholerae* strains carrying multiple virulence factors can be expected in the German Bight in future, as warm temperatures substantially accelerate their growth kinetics. Furthermore, we demonstrate that nutrient availability is significantly changing growth response of both *Vibrio* species towards salinity and temperature, especially leading to high population densities of *V. cholerae*. We therefore hypothesize that climatic changes such as extended warm periods and increased river discharge will likely give clinically *V. vulnificus* and *V. cholerae* the opportunity to survive and grow in more marine conditions of the German Bight in the future. Considering the North Sea as one of the fastest warming seas worldwide, microbial risk assessment is strongly recommended for the German Bight in future since an increase in *Vibrio* incidences is likely to happen. Despite similar growth patterns, *V. vulnificus* and *V. cholerae* showed differences in their kinetic response to changing environmental conditions, which makes a species-specific risk assessment of the environment crucial. Nevertheless, the growth behaviour of *V. cholerae* is less predictable in the German Bight, complicating monitoring and risk assessment attempts based solely on abundance analyses.

Acknowledgement

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Chapter III

Dangerous hitchhikers? Evidence for potentially pathogenic *Vibrio* spp. on microplastic particles

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Keywords: Synthetic polymers, Vector, Pathogens, North Sea, Baltic Sea

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Abstract

The taxonomic composition of biofilms on marine microplastics is widely unknown. Recent sequencing results indicate that potentially pathogenic *Vibrio* spp. might be present on floating microplastics. Hence, these particles might function as vectors for the dispersal of pathogens. Microplastics and water samples collected in the North and Baltic Sea were subjected to selective enrichment for pathogenic *Vibrio* species. Bacterial colonies were isolated from CHROMagar^{TM}Vibrio and assigned to *Vibrio* spp. on the species level by MALDI-TOF MS (Matrix Assisted Laser Desorption / Ionisation - Time of Flight Mass Spectrometry). Respective polymers were identified by ATR FT-IR (Attenuated Total Reflectance Fourier Transform - Infrared Spectroscopy). We discovered potentially pathogenic *Vibrio parahaemolyticus* on a number of microplastic particles, e.g. polyethylene, polypropylene and polystyrene from North / Baltic Sea. This study confirms the indicated occurrence of potentially pathogenic bacteria on marine microplastics and highlights the urgent need for detailed biogeographical analyses of marine microplastics.

Introduction

The production of synthetic polymers started over 100 years ago and meanwhile the worldwide production reached up to 311 million tons per year (PlasticsEurope, 2015). As a consequence of improper disposal synthetic polymers represent the most rapidly growing form of anthropogenic debris entering and accumulating in the oceans (Andrady, 2011, Thiel and Gutow, 2005).

Due to their durability most synthetic polymers are poorly degradable in the marine environment but become brittle and subsequently break down in small particles, so called microplastics (Andrady, 2011, Corcoran et al., 2009). Several size categorizations of plastics have been suggested by various researchers (Gregory and Andrady, 2003, Moore, 2008) while plastic fragments smaller than 5 mm are categorized as microplastics by Barnes et al. (2009). Once floating on seawater, plastic debris can be transported over long distances by wind, currents and wave action (Barnes et al., 2009).

As all surfaces in the marine environment microplastic is rapidly colonized by bacteria (Harrison et al., 2014) and subsequently by a plethora of organisms building up complex biofilms (Dobretsov, 2010). (Harrison et al., 2014) detected bacterial colonization of low density polyethylene microplastics already after 7 days exposure in marine sediments. Also (Lobelle and Cunliffe, 2011) proved biofilm formation on plastics after 1 week of incubation in seawater via quantitative biofilm assays. Prior studies evidenced that even harmful algal species were detected in biofilms on plastic debris (Masó et al., 2003). Being highly heterogeneous environments, biofilms offer important ecological advantages such as the accumulation of nutrients, as protective barrier, for mechanical stability (Flemming, 2002) or the formation of micro-consortia of different species that orchestrate the degradation of complex substrates (Wimpenny, 2000).

Zettler et al. (2013) showed that microbial communities on marine plastic debris differ consistently from the surrounding seawater communities and coined the term “Plastisphere” for this habitat. Furthermore, Amaral-Zettler et al. (2015) reported that “Plastisphere” communities are genetically unique from the free marine water communities that envelop them and possess dominant taxa that are highly variable and diverse. Moreover, the composition of biofilm communities on plastic in marine habitats varies with season, geographical location and plastic substrate type (Oberbeckmann et al., 2014a).

Zettler et al. (2013) have suggested that plastic particles may serve as vectors for the dispersal of human pathogens (*Vibrio* spp.). Using a culture-independent approach, the author's detected sequences affiliated to *Vibrio* spp. on marine plastic debris Zettler et al. (2013), i.e. on plastic particles in the North Atlantic by using molecular tools (Amplicon Pyrotag Sequencing). Furthermore, De Tender et al. (2015) recently detected Vibrionaceae on marine plastics from the Belgian North Sea, by using next-generation amplicon sequencing. However, due to short read lengths, a conclusive identification on the species level was not provided so far (De Tender et al., 2015, Zettler et al., 2013).

Species of the genus *Vibrio* belong to the class *Gammaproteobacteria* and are highly abundant in sediments, estuaries and marine coastal waters (Barbieri et al., 1999). *Vibrios* are gram-negative, rod-shaped chemoorganotrophic and facultatively anaerobic organisms. Besides occurring free-living in aquatic environments, *Vibrio* spp. are known to colonize a variety of marine organisms, utilizing released nutrients on these living surfaces (Huq et al., 1983, Visick, 2009) or living in symbiosis (McFall-Ngai and Ruby, 1991, McFall-Ngai, 2002, McFall-Ngai and Ruby, 1998).

Some *Vibrio* species are known as animal pathogens invading coral species and causing coral bleaching (Ben-Haim et al., 2003) and others are classified as human pathogens causing serious infections (Morris Jr and Acheson, 2003). Especially *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* are known as water-related human pathogens which cause wound infections associated with recreational bathing, septicaemia or diarrhea after ingestion of contaminated foods (Thompson et al., 2004a).

Although *Vibrio* infections are common in tropical areas, the last decade showed a significant increase in documented cases also in European regions, such as in the Mediterranean Sea (Gras-Rouzet et al., 1996, Martinez-Urtaza et al., 2005) or in the more temperate Northern waters (Eiler et al., 2006). Prior studies reported that the number of *Vibrio* infections correspond closely with the sea surface temperature pointing to a possible link to climate change related phenomena (e.g. global warming, heat waves) (Baker-Austin et al., 2010, Baker-Austin et al., 2013).

Böer et al. (2013) reported that *V. alginolyticus*, *V. parahaemolyticus, V. vulnificus and V. cholerae* occurred in water and sediments in the central Wadden Sea and in the estuaries of the rivers Ems and Weser. The most prevalent species were *V. alginolyticus* followed by *V. parahaemolyticus, V. vulnificus* and *V. cholera* (Böer et al., 2013), reflecting earlier findings on

the composition of *Vibrio* communities in other parts of the North Sea (Bauer et al., 2006, Collin and Rehnstam-Holm, 2011, Hervio-Heath et al., 2002, Schets et al., 2011). While *V. vulnificus* and *V. cholerae* were detected mainly in the Baltic Sea, *V. parahaemolyticus* occurred as the main potential pathogenic *Vibrio* spp. in the North Sea (Boer et al., 2013, Oberbeckmann et al., 2011b, Ruppert et al., 2004, Schets et al., 2010).

As already mentioned most synthetic polymers are poorly degradable and are rapidly colonized by microorganisms. Microplastics could be transported over long distances in marine environments, as compared to naturally occurring polymers, and therefore function as a vector for the dispersal of harmful or even human pathogenic species. To verify or falsify the occurrence of potentially pathogenic *Vibrio* spp. on marine plastics, we analysed plastics and corresponding water samples of the North and Baltic Sea with respect to potentially human pathogenic *Vibrio* spp. by using cultivation-dependent methods (alkaline peptone water (APW), CHROMagar™Vibrio), followed by state of the art identification of bacteria on the species level by MALDI-TOF MS (Erler et al., 2015). The main focus of the study was on detecting the main potentially human pathogenic species *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. Polymers were identified by ATR FT-IR (Attenuated Total Reflectance Fourier Transform - Infrared Spectroscopy).

Materials and Methods

Sampling

To detect *Vibrio* spp. attached to microplastics, neustonic particles were collected during two research cruises in 2013 and 2014 at 62 sampling stations in the North and Baltic Sea (see Table A1). Neuston samples were taken with a Neuston Catamaran equipped with a 300 µm net. The Catamaran was towed alongside the vessel for about 30 to 45 min per station. The volume passing the Neuston net was recorded by use of a mechanical flowmeter (Table A2). Further samples were taken at the drift line of the south port beach at the island Helgoland at low tide in August 2013 (station 63). Particles recovered in the cod end of the Neuston net or sampled at the drift line of Helgoland were sorted by stereo microscopy and using a Bogoroff chamber and finally transferred to Petri dishes containing sterile seawater. Single particles identified visually according to the definition by (Barnes et al., 2009) in a size range of 0.5 – 5 mm and to colour and texture as being synthetic polymers were picked with sterile forceps and washed three times with 10 ml of sterile seawater, to remove loosely attached organisms. For comparison of microplastic-attached and waterborne *Vibrio* spp., additional surface seawater samples were taken on both research cruises with a thoroughly flushed bucket or rosette sampler (SBE 911 plus, Sea-Bird Electronics, US) and a maximal volume of 1 l was filtered onto 0.45 µm sterile membrane filters (Sartorius stedim biotech, US). Environmental parameters (temperature, salinity) were recorded by a ship-based thermosalinograph (SBE 21SeaCAT, Sea-Bird Electronics, US) or by the sensors of the rosette sampler. The temperature of Helgoland was measured manually with a thermometer and the salinity was recorded with a salinometer (Autosal, GUILDLINE, Canada) (Table A3).

Enrichment & isolation of *Vibrio* spp.

All particles and membrane filters (seawater samples) were immediately transferred individually into sterile glass tubes with alkaline peptone water (15 ml APW) and incubated in a rotating incubator at 37 °C for 48 h in the dark for the growth of a broad spectrum of mesophilic and potentially pathogenic *Vibrio* spp., enabling their selective enrichment.
After APW incubation the tubes were visually checked for growth and turbid samples were plated by using an inoculation loop or Spiral-plater (easySpiral® Dilute; Interscience, France)

on selective CHROMagar™Vibrio (MAST Diagnostica GmbH, Germany) (Di Pinto et al., 2011). All inoculated CHROMagar™Vibrio were incubated at 37 °C for 24 h in the dark. The appearing colonies were checked with respect to distinct colony colorations typical for *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* according to the manufacturers' instruction. Representative colonies for each coloration were picked and differentially streaked out on marine broth agar (Oppenheimer and ZoBell, 1952) with reduced salinity (MB-50%=16PSU). Incubation was performed at 37 °C for 24 h in the dark.

Even though CHROMagar™Vibrio is a selective medium for the isolation of *V.* cholerae, *V. vulnificus* and *V. parahaemolyticus*, other species have the ability to grow on these media appearing with the same colony colorations. For instance, *V. fluvialis* occurred in mauve coloured colonies distinct from *V. parahaemolyticus* and *V. mimicus* in turquoise coloured colonies distinct for *V. vulnificus* and *V. cholerae*. Hence for a conclusive identification all presumptive *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* strains were further analysed by MALDI-TOF MS.

MALDI-TOF MS

For MALDI-TOF analysis, all isolates were grown overnight on MB-50% agar plates as described above. To create high quality mass spectra, proteins of the strains isolated during the cruise in 2013 were extracted using a previously described formic acid/acetonitrile extraction method (Mellmann et al., 2008). For fast identification, all other strains (cruise 2014 and Helgoland samples 2013) were analysed via the direct transfer procedure according to manufacturers` recommendations (Bruker Daltonics Inc., Germany, Bremen). This involved picking colonies after 24 hours of cultivation with sterile toothpicks and directly transferring onto the MALDI-TOF MS target plate (MSP 96 target polished steel) as thin layer. Each sample spot was first overlaid with 1 µl formic acid (70% v/v) followed by an overlay with 1 µl matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and directly screened. All spectra were acquired using the microflex LT/SH system (Bruker Daltonics Inc., Germany, Bremen). Species identification was done by using the Biotyper^{TM} software (version 3.1) according to the manufacturer's instructions, where 70 most prominent mass peaks were compared to the mass spectra of the Bruker library as well as the "VibrioBase" library (Erler et al., 2015).

In order to check the reliability of the species assignment via MALDI-TOF MS all *V. cholerae, V. vulnificus* and *V. parahaemolyticus* were verified by PCR amplification of species-specific genes and additionally screened for virulence-associated genes (section 2.4).

PCR of regulatory and virulence-related genes

As described previously (Oberbeckmann et al., 2011a), DNA extraction of *Vibrio* strains identified by MALDI-TOF MS was carried out using lysozyme/SDS lysis and phenol/chloroform extraction, followed by isopropanol precipitation. Prior to PCR experiments, DNA quantity and quality was determined photometrically (TECAN infinite M200, Switzerland). Species-specific PCR for toxR genes was performed with all *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* strains respectively using the universal forward primer *UtoxF* together with the species specific primers *VptoxR*, *VvtoxR* and *VctoxR*, respectively (Bauer and Rørvik, 2007, di Pinto et al., 2005). Specific PCRs targeting thermostable direct haemolysin (*tdh*) (Nishibuchi and Kaper, 1985) and the *tdh* related haemolysin (*trh*) (Honda et al., 1991, Honda and Iida, 1993)Honda and Iida, 1993) genes were performed with the primer sets *tdhD3F/tdhD* and *trhFR2/trhRR6* to strains assigned to *V. parahaemolyticus* (Bauer and Rorvik, 2007; Tada et al., 1992). To test *V. cholerae* strains for the presence of a unique chromosomal region indicating the serotypes O139 (Albert et al., 1997) and O1 (Katsuaki Hoshino 1998) and the cholera toxin gene *ctxA* (Singh et al., 2002) a multiplex PCR was performed with the primer sets *O139F/O139R, O1F/O1R* and *ctxA1/ctxA2* (Bauer and Rorvik, 2007; Mantri et al., 2006; Nandi et al., 2000). All reactions were performed in duplicate. In case of discordant results, a third PCR was carried out. The PCRs were performed as described by Böer et al. (2013) with the exception that 20 ng of template DNA was used. The following reference strains were used as positive controls: *V. vulnificus* ATCC 27562 (*VvtoxR)* (The Federal Institute for Risk Assessment, BfR), *V. parahaemolyticus* RIMD 2210633 (*VptoxR; tdh*) (German Collection of Microorganisms and Cell Cultures, DSMZ), V. parahaemolyticus CM12 *(tdh*; *trh*), *V. parahaemolyticus* CM24 (*trh*) (provided by Carsten Matz, HZI), *V. cholerae* CH 111 (*VctoxR*; *O1*), *V. cholerae* CH 187 (*VctoxR*; *O139*; *ctxA*) and *V. cholerae* CH 258 (*VctoxR*; *ctxA*; *O1*) (BfR). *V. harveyi* ATCC 25919 (DSMZ) was used as negative control in each PCR. PCR products were confirmed to be of the expected size by a MultiNA Microchip electrophoresis system (MCE-202 MultiNA, Shimadzu Biotech).

FT-IR analyses of particles

After incubation in APW, all particles were rinsed using deionized water and dried at 60°C overnight. Prior to analysis, particles were rinsed with ethanol (70% v/v) and the surface was scraped with a scalpel to avoid organic contamination interfering with FT-IR analysis. The FT-IR spectra of particles were recorded by the attenuated total reflectance (ATR) technique using a Tensor 27 spectrometer with a Platinum ATR unit (Bruker, Germany). For each analysis 16 scans in the range 4000-400 cm^{-1} with a resolution of 4 cm^{-1} and 6 mm aperture were performed and averaged. The obtained IR spectra were compared to reference-spectra of an in-house database covering 143 spectra of different synthetic polymers and the IR Library from Bruker Optics containing 350 entries. Spectra processing and database comparisons were performed by using OPUS 7.2. (Bruker, Germany).

Results

Occurrence and characterization of microplastics

Particles were collected from 39 stations in the North Sea and 5 stations in the Baltic Sea. In total, 170 particles were collected in the North Sea and 15 particles in the Baltic Sea, mostly abundant at stations 17, 56, 58 and 61, with ≥ 10 particles from each station, respectively (Table A4). Almost all particles showed signs of weathering, including cracks and pitting. Most particles were covered at least partially with dense biofilms on their surface, indicating colonization by various biota. Polymer identification of presumptive synthetic polymer particles, (ATR FT-IR (Table A3)) confirmed 141 as synthetic polymers, 14 particles were non-plastics such as chitin or keratin, and 30 could not be further identified. All of the 15 presumptive microplastics of Helgoland drift line were identified as synthetic polymers. The most abundant synthetic polymer throughout all sampling sites was polyethylene, comprising over 40 % of the collected particles at all sites. Polypropylene and polystyrene were also frequently found at all sites, representing 14-20 % and 5-7 % of all particles, respectively (Fig. 1).

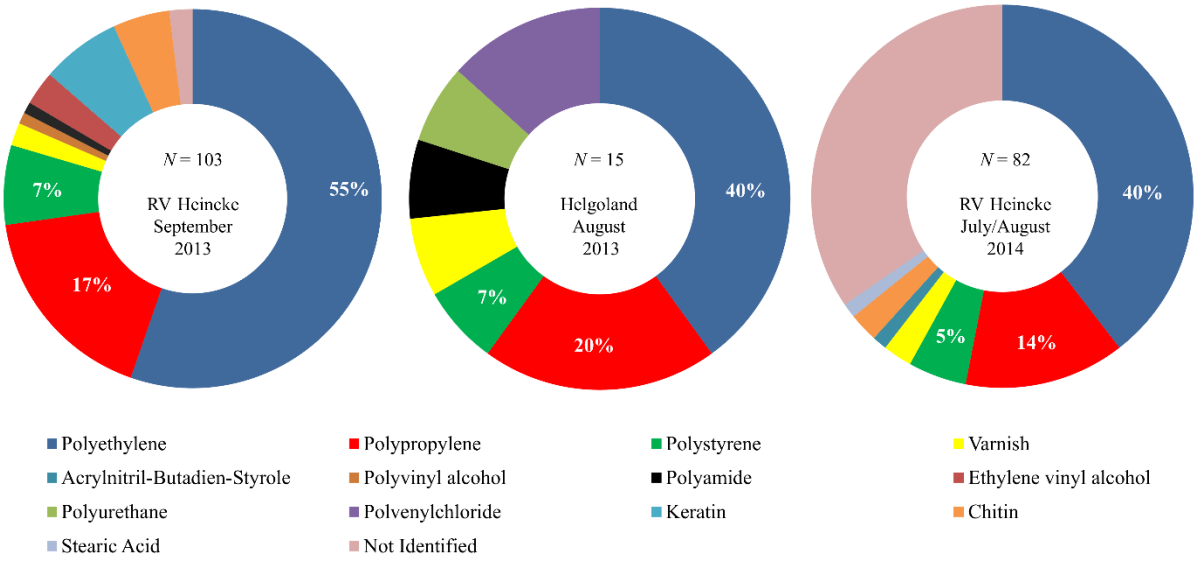

Figure 1: Proportions of synthetic polymers and other particles collected during research cruises in the North and Baltic Sea and the drift line of Helgoland. Sampling took place in September 2013 (left), July/August 2014 (middle) and July 2013 (right). Particles were characterized using ATR FT-IR spectroscopy. Also given are numbers of total particles (*N*) and percentages of polyethylene, polypropylene and polystyrene particles.

Identification and geographic distribution of *Vibrio* spp. in water samples

Water samples were taken from all stations in the North and Baltic Sea with the exception of Helgoland drift line (station 63) resulting in 326 APW enrichment cultures. Out of these, 323 displayed growth and were subjected to further isolation of bacteria on selective CHROMagar™Vibrio agar plates, with respect to *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus*.

From all water samples, 151 pure cultures of representative mauve and turquoise blue colonies were grown on marine broth agar and subjected to MALDI-TOF MS. Out of these, 104 were identified as *Vibrio* spp. by MALDI-TOF MS. With the exception of three isolates, all *Vibrio* water strains could be identified by MALDI-TOF MS on a conclusive species level. We identified 38 % out of all *Vibrio* water isolates (104) as *V. parahaemolyticus*, 16 % as *V. vulnificus* and 11 % as *V. cholerae*. Further on, 21 % of the strains were classified as *V. fluvialis*, 7 % as *V. mimicus*, 5 % as *V. diazotrophicus*, 1 % as *V. metschnikovii* (Table A6).

A single *V. parahaemolyticus* strain (VN-4212) isolated from water (station 3) carried the virulence-associated gene *tdh*, while *trh* was not detected in any strain (Table A6). No *V. cholerae* strain belonged to the *O1*/*O139* type or carried the *ctxA* gene.

In general, *V. parahaemolyticus* was detected only in North Sea waters (Fig. 2) in a temperature range of 14.9 to 21.1 °C and at salinities between 16.9 to 32.4 PSU (Table A3). The potentially pathogenic species *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* occurred mainly in coastal and estuarine regions of the North Sea. *Vibrio fluvialis* was the only species that was detected in open waters in the North Sea (Fig. 2 a, c). In the Baltic Sea both species, *V vulnificus* and *V. cholerae* appeared close to the Polish border at 14.5 to 14.9 °C and 5.7 - 7.3 PSU (station 36, 37, 38). *V. cholerae* occurred also nearby to Rostock at 14.1 °C and 11.7 PSU (station 31) (Fig. 2 b; Table A3). *Vibrio fluvialis* was detected once in Baltic surface water inside Germany and Denmark (station 32).

Identification and geographic distribution of *Vibrio* spp. on microplastics

All collected particles of North Sea, Baltic Sea and Helgoland drift line were subjected to selective APW enrichment resulting in 200 APW cultures. Out of these 161 displayed growth and were processed as described previously. From 15 microplastic particles from the North

and Baltic Sea, in total 37 putative (according to the colony colorations) *V. cholerae*, *V. vulnificus* or *V. parahaemolyticus* strains were isolated. At the drift line of Helgoland 4 putative *V. parahaemolyticus* strains from 4 different microplastic particles were isolated. Of these 41 strains, 22 were identified as *Vibrio* spp. by MALDI-TOF MS. Thirteen strains were identified as *V. parahaemolyticus* (59 %), six as *V. fluvialis* (27 %) and one as *V. alginolyticus* (5 %) (Table A5). Even though we isolated representative coloured colonies neither *V. vulnificus* nor *V. cholerae* could be detected on microplastic particles.

V. parahaemolyticus was isolated from three polyethylene fibres and four polyethylene fragments during the cruises in the North Sea at temperatures between 14.8 and 21.1°C and salinities between 12.6 - 32.4 PSU (Table A3). These were collected in the Ems estuary (station 5), near the uninhabited island Mellum (station 9), the Elbe estuary (station 21), and close to the Frisian islands (stations 39 and 41) (Fig. 2 a, c). Additionally *V. parahaemolyticus* was isolated from two polyethylene films and two polypropylene fragments of Helgoland drift line at a water temperature of 16.6°C and a salinity of 30.2 PSU (station 63) (Fig. 2 c). *V. fluvialis* was detected on four non-identified particles collected between the UK and the Netherlands (stations 58, 59) and on a polyethylene fragment of the Weser estuary (Germany, station 11). *V. alginolyticus* was detected on one polyethylene fragment close to the Frisian island Juist (station 41). In the English Channel (station 55) an unspecified *Vibrio* spp. was detected on a polyethylene fragment (Fig. 2 c).

One polypropylene film (station 30; Fig. 2b) collected close to the coastal regions of Wismar in the Baltic Sea at 14.8 °C and 12.6 PSU (Table A3) was colonized by both species, *V. parahaemolyticus* and *V. fluvialis*. *Vibrio parahaemolyticus* was detected only once on this single microplastic particle in the Baltic Sea (Fig. 2b).

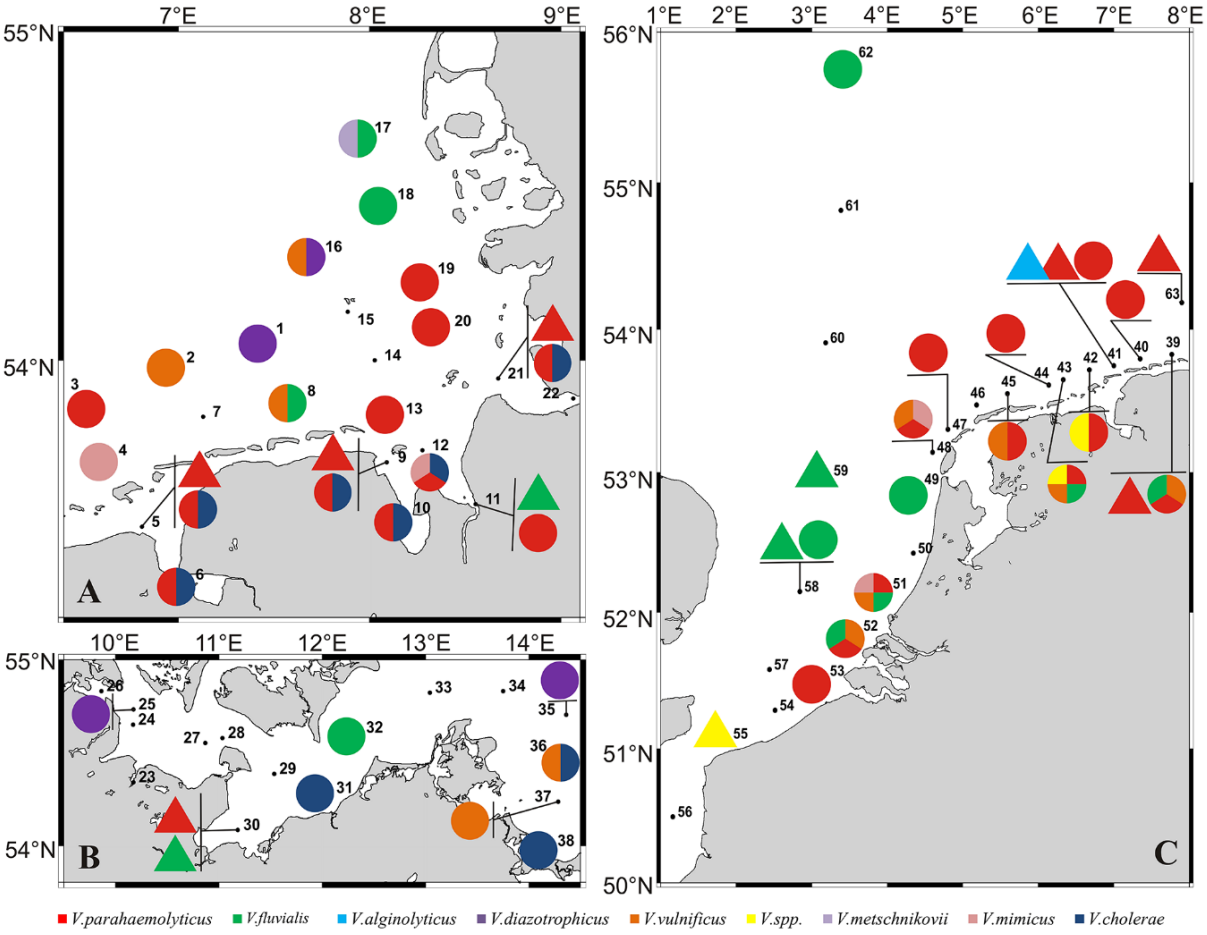

Figure 2: Geographical occurrence of *Vibrio* spp. On microplastics and surface water of a) the North Sea from research cruise HE409 on RV Heincke in September 2013 b) the Baltic Sea from research cruise HE409 on RV Heincke in September 2013 and c) North Sea from research cruise HE430 on RV Heincke in July/August 2014 and the drift line of Helgoland (station 63). (O) species detected from surrounding seawater (Δ) species detected on microplastic particles.

Discussion

Although the microbial colonization of marine plastic particles was reported already in the 1970s, (Carpenter et al., 1972, Carpenter and Smith, 1972) this issue received increasing attention in the last years due to the discovery of the large oceanic garbage patches (Kaiser, 2010, Ryan, 2014) and the general perception of microplastics being an emerging environmental topic of concern. In this context, it was also hypothesized, that microplastics may function as a vector for dispersion of invasive species including toxic algae but also pathogenic organisms (Masó et al., 2003, Zettler et al., 2013).

Recently the microbial community on marine plastics was targeted in several studies, highlighting the composition and diversity of plastic-attached microorganisms (Carson et al., 2013, Zettler et al., 2013, Oberbeckmann et al., 2014a, Reisser et al., 2014, Amaral-Zettler et al., 2015, De Tender et al., 2015). Within the microbial community on the "Plastisphere" (Zettler et al., 2013) sequences related to the genus *Vibrio,* a group of bacteria also containing serious pathogens, were found (De Tender et al., 2015, Zettler et al., 2013). However, in both studies a conclusive identification on the species level could not be provided so far due to the usage of next-generation amplicon sequencing and the short read lengths inherent to the methodology.

In our study we were able to prove the presence of potentially pathogenic *V. parahaemolyticus* on twelve floating microplastics for the first time by a selective cultivation approach and identification on species level by MALDI-TOF MS.

Microplastics in the North and Baltic Sea

In the present study, we observed more microplastic particles in North Sea waters compared to the Baltic Sea. Up to now, information on the abundance of microplastics in coastal waters of the North and Baltic Sea is scarce, and a comparison of the findings is problematic due to missing standard operational procedures (SOP) for sampling, extraction and analysis of microplastics (Löder and Gerdts, 2015).

During both cruises in 2013 and 2014, 77 % of all collected and identified microplastics as well as all collected microplastics at the drift line of Helgoland, occurred as fragments with rough and uneven edges clearly indicating a breakdown of larger plastics (Thompson et al., 2004c). Brittleness of particles including cracks and pitting could be detected on collected

microplastics which might be the result of degradation processes or wind and wave actions (Andrady and Neal, 2009). Thus, it could be suggested that most of the collected microplastics were exposed long enough to the marine environment to get brittle and be transported over long distances. Thiel et al. (2011) reported hotspots of accumulating microplastics in the North Sea and a rapid transport through the German Bight due to strong westerly winds. In contrast, based on the relationship between litter accumulation on Helgoland beaches and southerly winds, Vauk and Schrey (1987) suggested that these winds might push anthropogenic debris from source regions which results in accumulation on local beaches. Galgani et al. (2000) proposed that the predominant northward currents in the eastern part of the German Bight transport floating debris and accumulate it in an area to the west of Denmark. However due to the focus of our study (*Vibrio* spp.), these findings should be interpreted with care since we were not aiming at monitoring microplastics explicitly and in a systematic way.

By far the majority of microplastics from the North and Baltic Sea as well as from the Helgoland drift line was identified as polyethylene, followed by polypropylene and polystyrene (Fig. 1). Prior studies already reported high portions of these three polymers in the course of various samplings in marine and coastal environments which mirrors our results (Browne et al., 2010, Morét-Ferguson et al., 2010, Oberbeckmann et al., 2014b) and furthermore reflect the usage of these polymers in the worldwide economy. In the United States polyethylene, polystyrene, polypropylene and polyethylene terephthalate are the most widely produced and disposed synthetic polymers (Barnes et al., 2009). In Europe polyethylene and polypropylene are the synthetic polymers with the highest demand in various application segments, especially in packaging (PlasticsEurope, 2015).

Vibrio **hitchhikers**

Biofilm communities on environmental plastic samples were recently characterized in several studies applying molecular tools. The diverse microbial communities on marine plastic debris differed clearly from the surrounding seawater (Zettler et al., 2013, Oberbeckmann et al., 2014b, Amaral-Zettler et al., 2015, De Tender et al., 2015).

The herein described presence of potentially human pathogenic *Vibrio spp*. on microplastics has to be discussed in the light of these latter studies. The first indication of the presence of *Vibrio* spp. on marine microplastics was published by Zettler et al. (2013), who reported the dominance of this genus that constituted nearly 24 % of the whole biofilm community on a

single polypropylene particle collected from the North Atlantic. In 2015, De Tender et al. (2015) reported the detection of members of the family Vibrionaceae on marine plastics from the Belgian North Sea. Recently a review of Keswani et al. (2016) highlights the lack of knowledge about the persistence of potentially pathogenic *Vibrio* spp. on plastic debris. Our study clearly confirmed the presence of cultivable *Vibrio* spp. on 13 % of all marine collected microplastic particles. Amongst others, potentially pathogenic *V. parahaemolyticus* strains were detected on 12 microplastic particles. Only collected polyethylene, polypropylene and polystyrene fragments were colonized by *Vibrio* spp.

In general, *Vibrio* spp. tends to colonize marine biotic surfaces like corals or zooplankton / phytoplankton surfaces. *V. cholerae* strains, both *O1* and non-*O1* serovars, as well as *V. parahaemolyticus* strains were found to be attached to the surfaces of copepods in natural waters (Huq et al., 1983). In comparison to naturally occurring polymers like chitin, synthetic polymers are poorly degradable and could therefore function as a mechanism for the transport and persistence of *Vibrio* species. Pruzzo et al. (2008) reviewed substrate-specificity of *V. cholerae* on the naturally occurring polymer chitin. They reported close interactions between *V. cholerae* and chitin surfaces in the environment including cell metabolic and physiological responses e.g. chemotaxis, cell multiplication, biofilm formation, and pathogenicity. With respect to plastic microbial communities, Oberbeckmann et al. (2014b) found that the structure and taxonomic composition of these plastic associated communities vary with plastic type, but also with geographical location and season. Moreover, Amaral-Zettler et al. (2015) found that “Plastisphere” communities of the Atlantic and Pacific Ocean clustered more by geography than by polymer type, with exception of polystyrene that showed significant differences to polyethylene and polypropylene.

The substrate specificity of *Vibrio* spp. on synthetic polymers is still not investigated. However, since polyethylene, polypropylene, polystyrene and polyethylene terephthalate are the most widely disposed synthetic polymers globally (Barnes et al., 2009), it can be supposed that our results are biased due to the high accumulation of these specific synthetic polymers in our oceans.

Potentially pathogenic *V. parahaemolyticus* as well as *V. fluvialis* occurred in water as well as on microplastic particles. Recent studies report that *V. parahaemolyticus* and *V. alginolyticus* are prevailing inhabitants of North Sea waters (Böer et al., 2013, Oberbeckmann et al., 2011b). In contrast, *V. vulnificus* and *V. cholerae* are more abundant in the Baltic Sea (Böer et al., 2013),

which is also reflected by our findings. As already shown elsewhere, free-living bacterial communities in general differ significantly from plastic-attached ones (Amaral-Zettler et al., 2015, De Tender et al., 2015, Oberbeckmann et al., 2014b, Zettler et al., 2013), which holds also for microplastics investigated here. With respect to potentially pathogenic *Vibrio* spp., the species *V. vulnificus* and *V. cholerae* were only isolated from seawater samples but not identified on microplastics in the framework of our study. In contrast, *V. parahaemolyticus* was detected in both, water and on microplastic particles (Fig. 2). Additionally, *V. parahaemolyticus* was detected once in the Baltic Sea and only on a microplastic particle throughout the entire cruise.

Plastic is a persistent material and may serve as a reservoir and vector for potentially pathogenic microorganisms. The drift of potentially harmful algae species, barnacles and bryozoans on plastic debris (Barnes, 2002, Masó et al., 2003) is already well documented. Our results fuel the evidence for potentially pathogenic bacteria being dispersed on microplastic particles by wind or currents. However, although we identified *V. parahaemolyticus* on microplastics to species level, due to the high intra-species diversity information on the geographical origin of these hitchhikers or the microplastics is not possible, since the assignment of *Vibrio* species down to specific ecotypes was not successful.

Vibrio spp. on microplastics were detected mainly close to the coast and only occasionally offshore. However, microplastics and seawater samples carrying *V. parahaemolyticus* were located exclusively in estuarine and coastal areas of the North and Baltic Sea. *V. parahaemolyticus* occurrences in seawater were already addressed in several studies in Northern European waters (Bauer et al., 2006, Böer et al., 2013, Collin and Rehnstam-Holm, 2011, Ellingsen et al., 2008, Lhafi and Kuhne, 2007, Oberbeckmann et al., 2011b, Schets et al., 2011, Schets et al., 2010). Environmental parameters, such as temperature, salinity or plankton abundance have an effect on *Vibrio* spp. communities and abundances (Caburlotto et al., 2010, Blackwell and Oliver, 2008, Drake et al., 2007, Martinez-Urtaza et al., 2008, Thompson et al., 2004b, Turner et al., 2009, Vezzulli et al., 2009). Vezzulli et al. (2010b) and Schets et al. (2010) identified seawater temperature as a key factor influencing the presence of *Vibrio* spp., for instance it is well documented that *V. parahaemolyticus* favours warmer water temperatures (Sobrinho et al., 2010). Recently, pathogenic *V. parahaemolyticus* was detected even in temperate European waters (Baker-Austin et al., 2010, Martinez-Urtaza et al., 2008). Martinez-Urtaza et al. (2008) observed higher occurrence of this taxon during

periods of lower salinity and in general this taxon was primarily detected in areas of reduced salinity close to freshwater discharge runoff, which is also in agreement with our findings.

In our study *V. parahaemolyticus* occurred also on microplastics collected from the drift line at Helgoland. Oberbeckmann et al. (2011b) detected *V. parahaemolyticus* during summer months and reported that the abundance of *Vibrio* spp. was influenced by specific environmental conditions like the decrease in salinity due to an inflow of coastal water at Helgoland Roads (North Sea, Germany). Each *Vibrio* group was influenced by different combinations of environmental parameters but no single environmental parameter could explain the whole community structure of *V. alginolyticus* and *V. parahaemolyticus* populations in the German Bight (Oberbeckmann et al., 2011b). The authors also reported that free-living and plankton-attached *Vibrio* spp. abundances were mainly driven by the same environmental parameters (Oberbeckmann et al., 2011b). This suggests that the potentially pathogenic *V. parahaemolyticus* detected both on North Sea microplastics and in seawater samples of one station were influenced equally by environmental conditions.

Conclusion

This study successfully evidences the occurrence of potentially pathogenic *Vibrio* spp. on the species level on marine microplastics by use of MALDI-TOF MS for the first time. In most of the cases, these species co-occurred also in surrounding seawater, suggesting that seawater serves as a possible source for *Vibrio* colonization on microplastics. The fact that we for the first time detected *V. parahaemolyticus* exclusively on polyethylene, polypropylene and polystyrene particles, points to the urgent need to further address the biogeography and persistence of these hitchhikers on marine microplastics. Studies on the co-occurrence of specific *V. parahaemolyticus* genotypes on microplastic and surface water from the North Sea are particularly important specifically with reference to the potential health impacts of microplastic-colonizing microbial assemblages.

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General Discussion

The German Bight, located in the southeastern North Sea, is a unique coastal environment including the world's largest tidal flat area, which is strongly influenced by freshwater influx from the rivers Ems, Weser and Elbe, and is impacted by pronounced anthropogenic activities. So far, studies on the ecology of *Vibrio* spp. in this coastal region were mainly focused on the most abundant potentially pathogenic species *V. parahaemolyticus* and *V. alginolyticus* in marine regions ("Helgoland Roads") (Oberbeckmann et al., 2011a, Oberbeckmann et al., 2011b) and on the most prominent potentially pathogenic species *V. parahaemolyticus, V. vulnificus and V. cholerae* at recreational beaches at the Ems and Weser estuaries along the coastline (Böer et al., 2013). However, generally very little information is available on *V. vulnificus* and *V. cholerae* with respect to their spatio-temporal occurrence in the salinity gradient of the German Bight. Furthermore, little is known about the spectrum of virulence-associated genes of those species in this region while no investigations exist on the ability of clinically relevant strains to grow in the waters of the German Bight. The following sections discuss, in a general context, the contribution of this study to addressing those knowledge gaps. Finally, conclusions gathered in this study, an outlook and future recommendations are enclosed.

This thesis embodies the most comprehensive species-specific and spatio-temporal analysis of potentially pathogenic *Vibrio* occurrence, abundance and their pathogenic characterization in surface waters of the German Bight (Chapter I). Additionally, this study constitutes the first pathogen growth potential analysis to determine to which extent a spectrum of *in situ* environmental conditions would potentially support the growth of clinically relevant *V. vulnificus* and *V. cholerae* strains in the German Bight (Chapter II). The final chapter highlights for the first time the presence of cultivable potentially pathogenic *V. parahaemolyticus* on marine floating microplastics, which may serve as vector for dispersal or even as accumulation site for pathogenic species (Chapter III).

Driving forces of potentially pathogenic *Vibrio* spp. in the German Bight

Ecological driving forces of *Vibrio* spp. occurrence and abundance are reported to be species-specific in the environment (Takemura et al., 2014). Especially in coastal and estuarine environments, where physicochemical conditions are subjected to rapid spatio-temporal changes, the ecology of potentially human pathogenic *Vibrio* spp. is still poorly understood.

Previous approaches based on bacterial isolates lead to the conclusion that *V. parahaemolyticus* is a prevailing inhabitant of the North Sea (Böer et al., 2013; Oberbeckmann et al., 2011b) while in contrast, *V. vulnificus* and *V. cholerae* are more abundant in the Baltic Sea (Böer et al., 2013). Those findings are reflected in Chapter III of this thesis, also based on isolation methods (Kirstein et al., 2016). On the other hand, the spatio-temporal detection of potentially pathogenic *Vibrio* spp. based on MPN-PCR (Chapter I) revealed a mainly coastal presence of *V. cholerae* in low abundances (10^{-1} - 10^{0} MPN L^{-1}) and a seasonality of *V. parahaemolyticus* and *V. vulnificus* with high abundances throughout the salinity transect of the German Bight in summer (Figure 1.3). So far, studies reported accordingly pronounced seasonal patterns of *V. parahaemolyticus* and *V. vulnificus* in coastal recreational beaches of the German Bight while *V. cholerae* was detected only sporadically at coastal sites (Böer et al., 2013).

- **Increase in sea surface temperature**

Climate change affects coastal ecosystems by not only seawater warming, but also leads to a number of extreme events, including heatwaves and heavy precipitation. Focusing on the increase in sea surface temperature (SST), the North Sea is one of the most rapidly warming seas in the world (Emeis et al., 2015, Wiltshire et al., 2010, Belkin, 2009), which has been suggested to enhance the possibility of future blooms and outbreaks of *Vibrio* spp. (Vezzulli et al., 2016). This thesis highlights that the abundance of all three investigated *Vibrio* spp. in the German Bight is driven by temperature and revealed a species-specific response to increasing temperatures; *V. parahaemolyticus* has a linear relationship with temperature (Chapter I) while *V. vulnificus* and *V. cholerae* showed non-linear correlation patterns with greater effects at elevated temperatures (Chapter I + II). A particularly strong influence of temperature on *V. vulnificus in vitro* growth parameters (Chapter II) as well as on *in situ* abundances (Chapter I)

has been revealed. Altogether, it can be concluded that *V. vulnificus* growth is solely triggered by temperature, whereas other abiotic and biotic factors showed only minor effects. Accordingly, a broad meta-analysis on environmental predictors of *Vibrio* spp. abundances revealed that temperature explains most of the variance in *V. vulnificus* abundances and is usually a stronger predictor of high abundances than nutrients, salinity and biotic factors (Takemura et al., 2014). It has already been shown that long term (44 years) effects of seawater warming explains summer abundance variations of the genus *Vibrio* in the North Sea with up to 45 % (Vezzulli et al., 2012). These authors correlated the increase of the plankton-associated *Vibrio* abundances with climate-induced sea surface warming off the Rhine and Humber estuaries of the North Sea. However, *in situ* analyses of the German Bight (Chapter I) did not reveal a general increase in potentially pathogenic *Vibrio* spp. abundances between the first (2015) and second (2016) study period of Chapter I, nor in comparison to recent observations in this region by other authors (Table 4.1). A cultivation-dependent approach previously revealed concentration levels of up to 4.3 x 10^3 cells L^{-1} for free-living as well as plankton-attached *Vibrio* spp. in the German Bight (Oberbeckmann et al. 2011b) and suggested that both lifestyles are mainly influenced by the same conditions throughout the year. Consequently, investigations of potentially pathogenic *Vibrio* spp. in this thesis were conducted on surface water samples without a prior size fractionation, resulting in the analysis of the free-living as well as particle-attached potentially human pathogenic *Vibrio* spp. population in the German Bight (Chapter I).

Table 4.1: Potentially pathogenic *Vibrio* spp. abundances in European waters. MPN = Most probable number; TCBS = Thiosulfate-citrate-bile salts-sucrose agar; CFU = Colony forming units; PCR = Polymerase chain reaction; RT-PCR = Real-Time PCR;

Study Year	Detection Method	Study Area	Season	Sample type	V. spp.	V. p	V. v	V. c	Unit	Reference
2008	MPN, TCBS	USA (Great Bay Estuary)	summer	Water	-	-	-	150	MPN/l	(Schuster et al., 2011)
2008	MPN, TCBS	USA (Great Bay Estuary)	summer	Oyster	-	-	-	150	MPN/l	(Schuster et al., 2011)
2008 - 2010	TCBS, CFU	Helgoland Roads (North Sea)	year	Water	4300	-	-	-	CFU/l	(Oberbeckmann et al., 2011b)
2009 -2010	MPN, TCBS	Helgoland Roads (North Sea)	year	Mussels	240	-	-	-	MPN/g	(Oberbeckmann et al., 2011b)
2005 - 2006	MPN, PCR	Galicia, Spain (Offshore Atlantic)	year	Zooplankton	-	110	-	-	MPN/g	(Martinez-Urtaza et al., 2012)
2009 - 2011	MPN, ChromAgar™Vibrio	Estuarine Beaches (North Sea)	year	Water	-	63000	460	50	CFU/l	(Boer et al., 2013)
2009 - 2011	MPN, ChromAgar™Vibrio	Estuarine Beaches (North Sea)	year	Sediment	-	1600	48	0,07	CFU/g	(Boer et al., 2013)
2011	MPN, RT-PCR	French coastal lagoon (Mediterranean Sea)	rainy season	Water	-	1000	2100	93	MPN/l	(Esteves et al., 2015c)
2007 -2013	MPN, Culture	USA (Great Bay Estuary)	year	Oyster	-	4600	-	-	MPN/g	(Urquhart et al., 2016)
2015 - 2016	MPN, PCR	North Sea (Elbe Estuary)	year	Water	-	2400	4600	24	MPN/l	This thesis

❖ **Extended warm seawater periods**

Not only temperature itself has an effect on *Vibrio* spp. but also the duration of the warm water period (> 17 °C) heavily influenced the occurrence of potentially pathogenic *Vibrio* spp. in the German Bight (Chapter I). Similarly, extended warm periods have been shown to provide a basis for longer occurrences of viable potentially pathogenic *Vibrio* spp. in the Sydney Harbour estuary, Australia (Siboni et al., 2016) and an increased frequency of potentially pathogenic *Vibrio* spp. in an east Canadian estuary (Banerjee et al., 2018). *In vitro* growth of clinically relevant *Vibrio* strains showed significantly shorter lag times above 17 °C (Chapter II), explaining the prolonged *in situ* detection of *Vibrio* spp. above this seawater temperature (Chapter I). The trend of extended warm periods for the North Sea is estimated to increase further with climate change (Brennholt et al., 2014). This likely will further increase the exposure and infection risk with *V. vulnificus* in the German Bight in future, as suggested by Semenza et al. (2017) for *V. vulnificus* infections in European countries.

❖ **Frequent heatwave events**

Climate change has led to an increase in extreme climatic events like heatwaves and heavy precipitation in the past half century (Stocker et al., 2013) and it is very likely that those events will become more frequent in the future (Christidis et al., 2015). Those extreme events have been linked to an increase in infection reports associated with potentially pathogenic *Vibrio* spp. in northern European countries. Previous studies reported a heatwave-associated increase of *Vibrio* infection cases in European temperate regions (Baker-Austin et al., 2016), especially when SST exceeded 18 °C (Baker-Austin et al., 2013). Chapter II demonstrated a strong temperature dependency of clinically relevant *V. vulnificus* and *V. cholerae* growth kinetics, with particularly high specific growth rates and very low lag times above 17.5 °C. Given that heatwave events in Europe, that would occur twice a century in the early 2000s, are now expected to occur twice a decade (Christidis et al., 2015), peaks in *Vibrio* infection cases can be expected in the German Bight in future and a regular monitoring is inevitable.

❖ **Frequent heavy river discharge events**

Besides temperature, salinity is known to be the second most prominent factor influencing total *Vibrio* populations while its effect on single species varies in importance (Takemura et al., 2014). Particularly in estuarine regions, which represent highly dynamic interfaces between fresh and marine waters, *Vibrio* spp. need to be highly adapted to temporal and spatial physico-chemical fluctuations. *V. parahaemolyticus* and *V. vulnificus* were present throughout the salinity gradient during the warm water season, showing a high adaptability to elevated salinities (Chapter I). Although varying reports are present for the influence of salinity on *Vibrio* abundance in previous studies (Johnson, 2015, Takemura et al., 2014) as well as in this thesis, Chapter I revealed a peak of *V. parahaemolyticus* and *V. vulnificus* abundances during a heavy river discharge event depicted by increased nutrients and a seawards shifted salinity gradient. Those *in situ* investigations were underlined by *in vitro* assays in Chapter II in which no growth parameters of *V. vulnificus* and *V. cholerae* showed a salinity dependency in seawater samples (PGP) while growth under high nutrient conditions was mainly influenced by salinity, especially regarding population densities (Figure 2.3). High *Vibrio* abundances and changes in the overall *Vibrio* community associated with nutrient-rich freshwater influx in

coastal environments have been addressed previously (Jesser and Noble, 2018, Esteves et al., 2015b). Consequently, the expected frequent heavy rainfall events in future and with those associated increased river discharge (van Vliet et al., 2013) are likely to support growth of human pathogenic *Vibrio* spp. in the German Bight, probably leading to acute exposure and increased occurrence of infection cases in this region.

- **Impact of changing nutrient concentrations**

This thesis enabled a comparison of the fundamental niche, *in vitro* growth (PGP) of clinically relevant *V. vulnificus* and *V. cholerae* strains (Chapter II) and *in situ* abundances of potentially pathogenic *V. vulnificus* and *V. cholerae* in the German Bight (Chapter I). Concurrent with Materna et al. (2012), this thesis demonstrated that the realized ecological ranges (Chapter I & II) of *V. vulnificus* and *V. cholerae* strains represent only a segment of their actual fundamental niches, in which these species are able to grow *in vitro* without nutrient limitation (Chapter II).

In Chapter I of this study, NO_2 is found to be an important driver of *in situ Vibrio* abundances in the German Bight (Hackbusch et al., 2020). While previous studies found that nutrient levels determine potentially pathogenic *Vibrio* spp. in the German Bight (Oberbeckmann et al., 2011b, Böer et al., 2013), few studies reported a direct influence of nitrogen levels on potentially pathogenic *Vibrio* spp. abundances in estuarine environments (Oberbeckmann et al., 2011b, Blackwell and Oliver, 2008). Recently, Froelich et al. (2019) detected a nitrogen-related increase in total *Vibrio* spp. abundances in a long-term monitoring study of the Neuse River estuary. Considering metabolism of inorganic nitrogen compounds, genomic sequence analysis revealed that *Vibrio* spp. contain nitrate reductase coding genes (Grimes et al., 2009a), while other studies only reported an inorganic nitrogen metabolism of *V. cholerae* under hypoxic alkaline (Bueno et al., 2018) or glucose-limited conditions (Macfarlane and Herbert, 1982). Since those conditions (hypoxic alkaline, glucose-limited) were not prevalent in this study (Chapter I), the detected NO_2-dependency might be a proxy for collinear environmental drivers such as salinity or other nutrients such as phosphate or carbon in the coastal system. The biogeochemical nutrient cycles in marine and estuarine systems are complex (Dähnke et al., 2010, Brockmann et al., 1990), and so it can be difficult to identify multivariate associations of spatially and temporally varying parameters. Due to excessive

multicollinearity of physicochemical factors in the German Bight (Figure 4.3; Lucas et al. (2016)), representative variables were selected *a priori* for a statistical analyses (GAMM) in Chapter I. However, principal component regression (PCR) in Chapter II enabled the investigation of multiple predictors of growth parameters simultaneously overcoming the obstacles of multicollinearity among physico-chemical variables. Those PCRs revealed that inorganic nitrogen (PC 3: nitrate and ammonium) has an influence on the population density of the clinically relevant *V. cholerae* strain. Overall, the positive association of potentially pathogenic *Vibrio* spp. abundances with nutrient concentrations detected in this study is in agreement with studies reporting high nutrient loads as drivers of spatial variations and elevated *Vibrio* spp. abundances in coastal environments (Eiler et al., 2007, Böer et al., 2013, Oberbeckmann et al., 2011b).

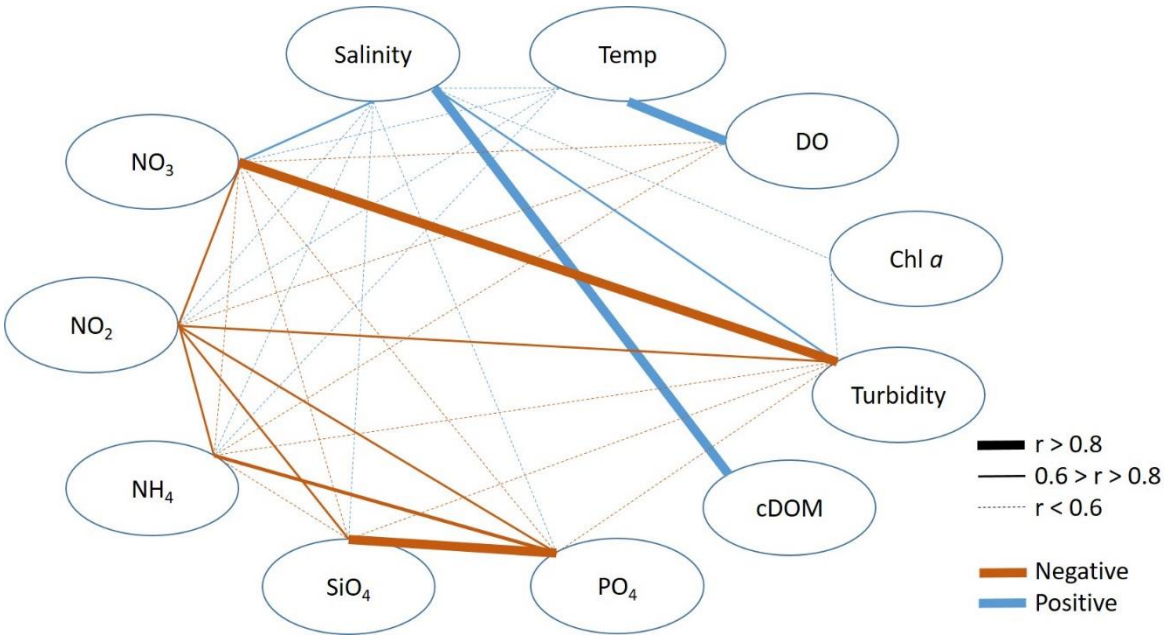

Figure 4.1: Multicollinearity of environmental parameters based on Spearman rank order correlations

❖ **Other putative factors influencing potentially pathogenic *Vibrio* spp.**

V. vulnificus in situ abundances detected in the German Bight highly correlated with all growth parameters observed during *in vitro* PGP assays (Figure 4.1), representing only the abiotic parameters (temperature, salinity, nutrients) of the German Bight. This leads to the conclusion that this species is relatively independent from biotic factors in this region, is able to thrive in

a planktonic lifestyle and is able to grow purely on dissolved compounds of the seawater samples. *V. cholerae in situ* abundance on the other hand correlated solely with the *in vitro* maximum specific growth rate (*rs* = 0.36 p < 0.01) (Figure 4.2). The high population densities of this species reached during *in vitro* growth (Chapter II) suggested high *in situ* abundances under the same abiotic conditions in spring and autumn samples (Chapter I). However, this was not detected by *in situ* studies (Figure 4.2), leading to the conclusion, that abundances of *V. cholerae* are highly influenced by environmental determinants that were not accounted for in the *in vitro* PGP assays. PGP assays were conducted with pure cultures by investigating solely the effect of physico-chemical parameters of the surface water samples. Hence, these assays did not cover predation, phage interactions, inter- and intraspecific competition and attachment to surfaces, which constitute important influencing factors on the ecology of *Vibrio* spp.. Exemplarily, it has been reported that besides having a free-living lifestyle, *V. cholerae* is using several survival strategies like the colonization of biotic (especially chitinous) surfaces to enhance their persistence especially as a response to nutrient-limitation (Yildiz and Visick, 2009, Tamplin et al., 1990). The lacking opportunity of attachment in PGP assays might explain, why the elevated abundances detected *in situ* could not be reproduced during *in vitro* studies. Other explanations for discrepancies between *in situ* abundances and *in vitro* growth might be that pathogenic *Vibrio* spp. *(V. cholerae, V. parahaemolyticus*) in the marine environment might be inhibited by other environmental *Vibrio* species (*V. splendidus, V. tasmaniensis and V. crassostreae*) due to intraspecific competition (Burks et al., 2017). It has also been shown that trophic regulation, such as protozoan predation (Worden et al., 2006) and lysis by bacteriophage (Jensen et al., 2006), has a significant role in controlling *V. cholerae* proliferation, limiting its population density in coastal waters. To ensure the exclusion of autochthonous bacteria and viruses, which might influence the growth behaviour of the *Vibrio* spp. strains, the seawater samples were treated by a combined pasteurization–filtration procedure (Vital et al., 2008) in the PGP experimental setup of Chapter II.

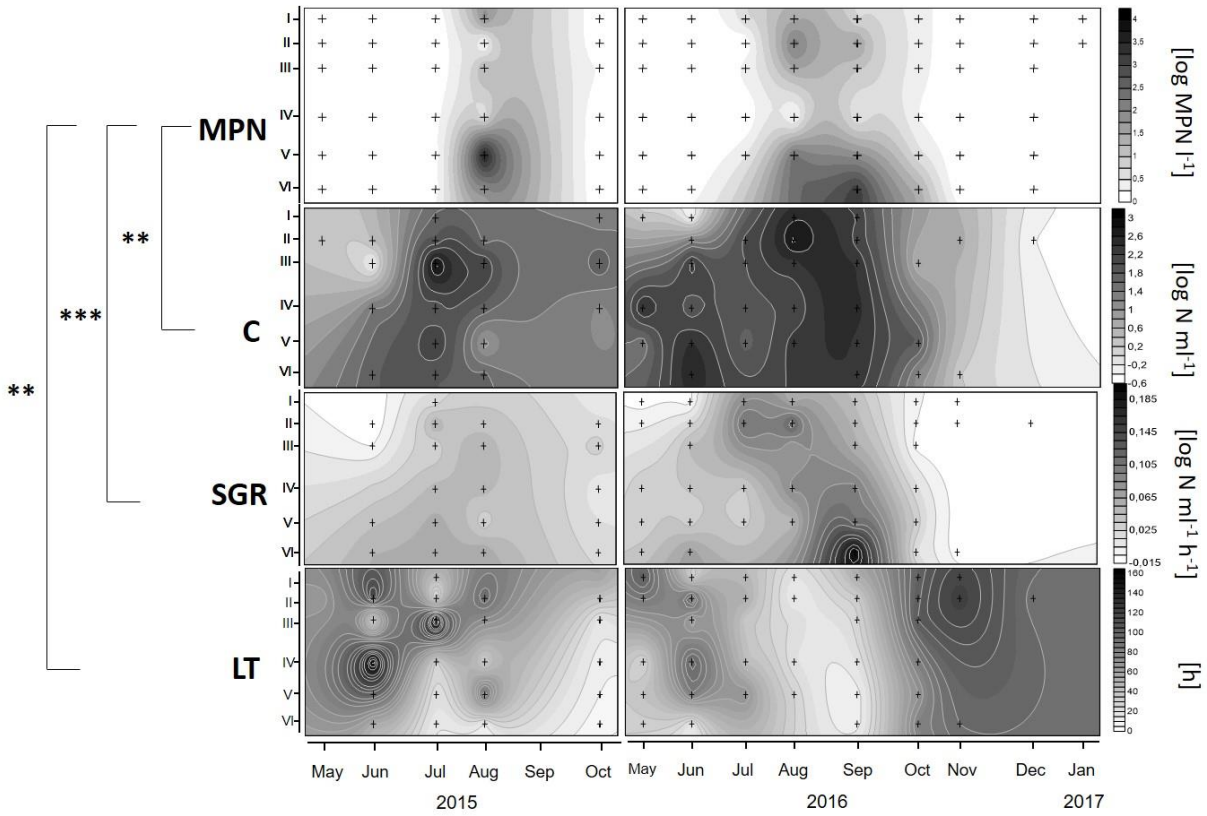

Figure 4.2: Spatio-temporal *V. vulnificus in situ* abundances in surface waters of the German Bight (MPN) and *in vitro* growth parameters in seawater samples revealed by pathogenic growth potential (PGP) assays. Following growth parameters are displayed: Population density (C), maximum specific growth rate (SGR) and lag time (LT). Significant spearman rank order correlations among *in situ* abundances and PGP growth parameters are given (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

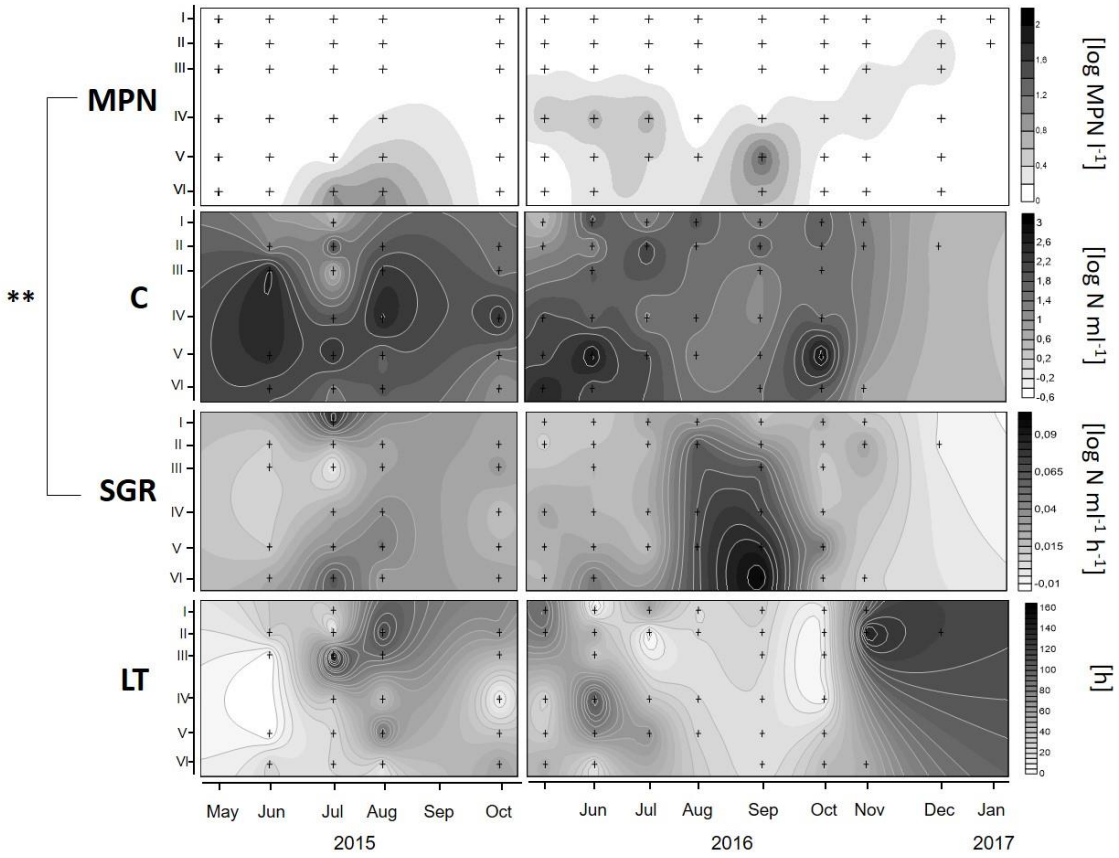

Figure 4.3: Spatio-temporal *V. cholerae in situ* abundances in surface waters of the German Bight (MPN) and *in vitro* growth parameters in seawater samples revealed by pathogenic growth potential (PGP) assays. Following growth parameters are displayed: Population density (C), maximum specific growth rate (SGR) and lag time (LT). Significant spearman rank order correlations among *in situ* abundances and PGP growth parameters are given (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

Using an ecological niche modelling approach based on published reports of the last half century, Escobar et al. (2015) stated that the North Sea is one of the most likely areas for *V. cholerae* incidences under current and future climate scenarios. Overall, this thesis substantially contributes to the understanding of the species-specific niche of potentially pathogenic *Vibrio* spp. in the German Bight and highlights the importance of a regular and systematic surveillance in future.

Vibrio spp. virulence factors in the German Bight on a spatio-temporal scale

Being transmitted through the faecal-oral route, outbreaks caused by toxigenic *V. cholerae* are most commonly reported in tropical regions accompanied by poverty, poor sanitation and inadequate or no drinking water treatment facilities, leading to severe epidemics (Baker-Austin et al., 2018, Faruque et al., 1998). This is not the case in northern Europe, thus the infrequent gastrointestinal infections with toxigenic *V. cholerae* are mostly imported from travels to Cholera-endemic countries (Schirmeister et al., 2014). Certainly, infection cases caused by various *Vibrio* spp. de facto originating in Europe, rather comprise extraintestinal infections like wound infections or otitis due to contact with contaminated water. Reports of *Vibrio* spp. infections in northern Europe are not officially notifiable but still seem to increase with time and have become more frequent over the last decade (Table 4.1). However, these data should be interpreted with caution as techniques for identifying pathogenic *Vibrio* infections have also improved. Nevertheless, since 2002, yearly incidences of contact-based *Vibrio* spp. infections in German coastal waters fluctuated between 0 - 20 cases, with most cases occurring in the warmer summers 2003, 2006, 2010 and 2018 (Robert Koch Institute, www.rki.de; status: 27.08.2019). To date, those infections are reported to be more common in the Baltic Sea than in the North Sea (Huehn et al., 2014).

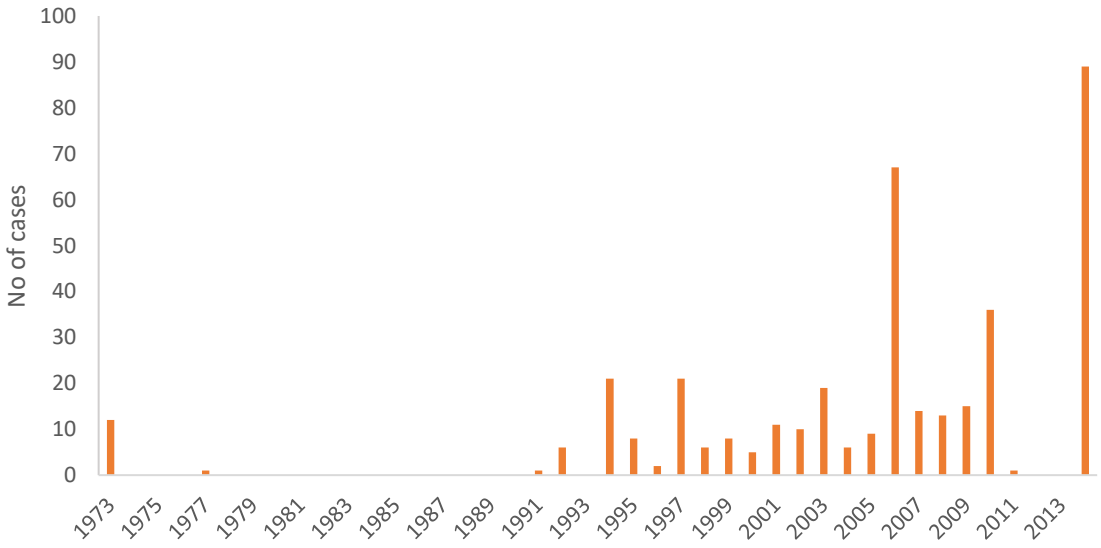

Figure 4.4: Annual frequency of infection cases reported in northern Europe caused by potentially pathogenic *Vibrio* spp. between 1973 and 2014. A detailed table in enclosed in the Supplemental material (Table S.4.1)

❖ **Virulence-associated genes of potentially pathogenic *Vibrio* spp. in the German Bight**

V. parahaemolyticus was found to occasionally carry the *trh* gene in the German Bight, concurrent with previous studies reporting the occasional presence of the *V. parahaemolyticus* genotype tdh^-/trh^+ in the German North Sea (Böer et al., 2013, Huehn et al., 2014). Nevertheless, its occurrence from coastal to offshore regions and over the entire studied temperature range from 7 to 20 °C is remarkable.

The reason for the absence of *V. vulnificus* infection cases originating from the North Sea has previously been suggested to be due to the relatively rare occurrence, low abundance and the preference of low-salinity environments of *V. vulnificus* (Bier et al., 2015). Opposed to that, Chapter I showed that *V. vulnificus* comprising the three pathogenic markers *nanA*, *manIIA* and PRXII is widely present across the entire study area in the German Bight. This is further reinforced in Chapter II, which revealed that the clinically relevant environmental *V. vulnificus* strain (vcg type C*, $nanA^+$, $manIIA^+$*) has the potential to grow *in vitro* in most examined surface waters at *in situ* temperatures in a salinity gradient of the German Bight. Given its highly temperature-driven growth kinetics and population density (Chapter II), frequent blooms of clinically relevant *V. vulnificus* can be expected in the rapidly warming North Sea (Emeis et al., 2015, Wiltshire et al., 2010) with extended warm water periods in the future (Brennholt et al., 2014).

This study revealed for the first time the presence of non-toxigenic *V. cholerae* serotype O139 genes in surface waters of the German Bight (Chapter I). The very limited spatio-temporal appearance of this gene and being never detected before in this region, indicates a unique and confined transport rather than a genetic acquisition, as discussed already in Chapter I. Possible transport routes are shipping traffic (Ng et al., 2018, Dobbs et al., 2013), long-distance migratory water birds (Fu 2019) and floating marine debris (Kirstein et al., 2016). The potential of marine microplastics to serve as vectors for potentially pathogenic *Vibrio* spp. has been demonstrated in Chapter III (Kirstein et al., 2016). These findings were recently confirmed by another study in the Baltic Sea, while it was discovered that regarding the enrichment of potentially pathogenic *Vibrio* spp., microplastics might play a rather minor role in the water column than naturally occurring surfaces such as wood or chitin (Oberbeckmann et al., 2018). Nevertheless, in comparison to naturally occurring surfaces, synthetic polymers

are poorly degradable and could therefore aid the transport and persistence of pathogenic *Vibrio* species over longer distances.

The major virulence factors Cholera Toxin (CT) and toxin co-regulated pilus (TCP) genes were not present in the German Bight, supporting the reports of *V. cholerae* being commonly non-toxigenic in this area (*ctxA*$^{-}$*/tcpAET*$^{-}$) (Schuster et al., 2011, Brennholt et al., 2014). While the pathogenicity of toxigenic O1 and O139 *V. cholerae* strains are well investigated, the pathogenic mechanisms of opportunistic non-O1/non-O139 *V. cholerae* pathogens are not as clearly understood. Infection cases caused by non-O1/non-O139 *V. cholerae* strains sporadically occur in northern European countries (Figure 4.4; Supplementary material Table S4.1). In this study, a variety of accessory virulence-associated genes of *V. cholerae* was detected, especially the El Tor haemolysin (*hlyAET*) and the acyltransferase of the RTX cluster (*rtxC*) were frequently detected and were spatio-temporally well distributed throughout the German Bight, possibly representing the endemic population. Similarly, Schwartz et al. (2019) reported that all environmental non-O1/non-O139 isolates from German coastal waters possessed the virulence-associated genes *hlyAET* and *rtxC.* Additionally, further accessory virulence factors with clinical importance were detected in this thesis (Chapter I); The Vibrio 7th pandemic island II (VSP-II), which is commonly found in toxigenic strains (Chatterjee et al., 2009, Rahman et al., 2008), the Type-III secretion system (TTSS), known to enhance the virulence of nontoxigenic *V. cholerae* strains (Zeb et al., 2019), and the Cholix Toxin (*chxA*), suggested to be associated with human extraintestinal infections (Awasthi et al., 2013). Overall, this study highlights that environmental human pathogenic *Vibrio* spp. comprise a reservoir of virulence-associated genes in the German Bight and possess a prerequisite for the infection of susceptible human hosts.

- ***Vibrio* spp. population diversity regarding virulence-associated genes**

It is unlikely, that virulence-associated genes detected in the German Bight (Chapter I) stem from single strains but rather display the population diversity of each targeted species. Several indications for different *Vibrio spp.* populations regarding virulence-associated genes were found in this thesis. In case of *V. vulnificus*, two genes were PCR targeted for the detection of the genomic island Pathogenicity Region XII (PRXII), while the detection of the "PRXIIpres" gene indicated to PRXII presence and the detection of the flanking region "PRXIIabs" showed

its complete absence. PRXIIabs is expected to be PCR amplified when *V. vulnificus* lacks the entire PRXII genomic island (Cohen et al., 2007). The presence of samples containing both genes (PRXIIpres$^+$ / PRXIIabs$^+$) indicates a mixed *V. vulnificus* population, including organisms harbouring PRXII and some lacking the entire genomic island.

In case of *V. cholerae*, spatio-temporally varying virulence profiles were detected, underpinning the great genetic diversity of environmental *V. cholerae* populations (Faruque et al., 2004, Chapman et al., 2015) especially with an increased co-occurrence in virulence-associated genes when warm temperatures prevailed (> 16.8 °C) (Figure 1.7). Yet, the *V. cholerae* Serotype O139 occurred only together with the virulence-associated genes *hlyAET*, *rtxC*, *VSP-II* and TTSS, exclusively, representing a high-risk virulence profile (Chapter I). Kirchberger et al. (2016) suggested that ecological differentiation of *V. cholerae* might occur at the subspecies level indicated by divergent clonal complexes and pronounced spatial distribution patterns, potentially occupying various ecological niches (Kirchberger et al., 2016). A pronounced niche specialization of *V. cholerae* was detected for the accessory virulence-associated traits Type-III Secretion System (TTSS) and the Cholix Toxin (*chxA*), displaying two distinct populations (Figures 1.4 & 1.7). The TTSS-population occurred mainly at coastal locations during warm water seasons correlating with various environmental parameters (turbidity, chl a, NO_3, PO_4) in this thesis. These findings support the hypothesis that this secretion system might contribute to the adaptation and enhanced fitness of environmental *V. cholerae,* as stated previously by Dziejman et al. (2005). Similarly, a Type-III Secretion System of *V. parahaemolyticus* (TTSS-2) has been hypothesized to enhance environmental persistence by facilitating the invasion of TTSS-2$^+$ strains into a coastal plankton community due to cytotoxicity and facultative parasitism on coexisting protists (Matz et al., 2011). *V. cholerae* TTSS is found in all diarrheal cases while being absent from other contact-based infection cases in German and Austrian patients (Schirmeister et al., 2014, Schwartz et al., 2019) and has been demonstrated to enhance the virulence of non-O1/non-O139 *V. cholerae* strains (Zeb et al., 2019). The *chxA*-population, rather present at marine stations in a broad temperature range, seems to give survival advantage to *V. cholerae* in offshore regions (Chapter I). This accessory virulence-associated gene is suggested to be associated with human extra-intestinal infections (like septicaemia) rather than enterotoxicity (Awasthi et al., 2013). In contrast to the high spatio-temporal genetic diversity of *V. cholerae* populations, this thesis strikingly revealed a unique virulence profile (*rtxC*$^+$, *hlyAET*$^+$, TTSS$^+$) at the Elbe river

mouth during the warm water season, indicating a highly adapted *V. cholerae* population to the estuary. Similarly, Gong et al. (2018) discovered distinct estuary-specific genes of non-O1/non-O139 *V. cholerae* strains in the Yangtze River estuary in China via genome analysis, displaying a unique environmental adaptation (Gong et al., 2018).

In this thesis, *V. cholerae* populations exhibited similar virulence profiles to clinical *V. cholerae* strains previously isolated from patients in Germany in terms of virulence-associated genes in the German Bight (Schwartz et al., 2019), making new infections highly possible. Environmental *V. cholerae* needs to be considered as a risk to human health in the German Bight (Chapter I) although the actual pathogenicity cannot be estimated only by the presence of these virulence-associated genes in the environmental population (Schwartz et al. (2019). However, epidemic *V. cholerae* strains evolved from environmental non-pathogenic strains by the acquisition of virulence genes through lysogenic bacteriophages (CTXPhage; Cholera Toxin) or through genetic elements transported through horizontal gene transfer (HTG) (Mekalanos, 2012, Hazen et al., 2010). A recent study detected lysogenic bacteriophages in environmental *V. cholerae* strains (Garin-Fernandez and Wichels, 2019). Overall, it can be concluded that an emergence of highly pathogenic *V. cholerae* strains in the German Bight become more likely.

Conclusion

This thesis profoundly contributes to the currently still limited knowledge and understanding of potentially pathogenic *Vibrio* spp. in the German Bight. On a species level, this study successfully determined environmental factors influencing *Vibrio* abundances as well as their virulence-associated gene occurrence. It could be demonstrated that not only increasing sea surface temperature in the German Bight will affect *Vibrio* spp. abundance and diversity in future, but also increasing environmental variability itself, such as prolonged warm water periods and increased river discharge together with related nutrient fluctuations. The spatio-temporal pathogenic characterization of potentially pathogenic *Vibrio* spp. highlighted the genetic diversity and niche-adaptation of *Vibrio* subpopulations in the German Bight, particularly for *V. cholerae* populations in coastal areas. This thesis comprises the most detailed *in vitro* growth analysis of clinically relevant *V. vulnificus* and *V. cholerae* strains in a broad range of physico-chemical conditions and showed their potential to grow in the German Bight. Additionally, the possibility of marine floating microplastics to serve as a vector for dispersal or even as an accumulation site for pathogenic *Vibrio* species was demonstrated in this thesis. Therefore, the growing presence of plastic litter in the marine environment may have consequences for human and animal health by the accumulation and transport of pathogens. We are convinced that the findings yielded by this study will help estimating and predicting *Vibrio* growth in the German Bight and serves as a basis for further systematic studies of potentially pathogenic *Vibrio* spp. responses on environmental fluctuations.

This thesis highlights that short-term changes, such as nutrient pulses and extreme heat waves can contribute to the occurrence of *Vibrio* blooms, as can slow but significant long-term temperature changes in the German Bight lead to increasing abundances and pathogenic occurrences. We highly recommend a microbial risk assessment and regular detection and monitoring programs for potentially pathogenic *Vibrio* spp. in the German Bight, since an increase in *Vibrio* incidences is likely to happen in future.

Conclusion

Outlook

This study highlights the importance of covering intra-specific genetic variability of potentially pathogenic *Vibrio* spp. in the German Bight. *Vibrio* community composition and the temporal variation in the relative abundance of virulence-profiles should be addressed in this region to examine if transient blooms of specific genotypes with greater infective risk could dominate coastal populations. High-resolution studies are needed to investigate small-scale responses of potentially pathogenic *Vibrio* spp. abundances to sudden changes of environmental conditions, such as heatwaves and increased freshwater influx. Future research approaches should include investigations of the physiological and morphological changes of pathogenic *Vibrio* spp. strains as a response to changing environmental conditions. Especially the assessment of the growth behaviour under future climate scenarios for a variety of pathogenic strains is needed.

To date, there is a lack in regular *Vibrio* monitoring as part of the German environmental surveillance program. Due to the increase of reported *Vibrio*-associated illnesses in Europe (Table 4.1) and based on the findings of this thesis, a regular seawater monitoring of abundances, common toxins and virulence-associated genes of the potentially pathogenic species *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* should become a priority. Besides, it is recommended to consider shellfish a relevant measure for the public health concerns surrounding in the German Bight given that shellfish consumption is one of the important exposure routes for infections. Additionally it is important to monitor areas with direct human contact such as recreational beaches, fisheries and aquaculture sites to estimate the risk of wound infections. Thus, high spatio-temporal resolution monitoring would help to elucidate community shifts particularly of *V. cholerae* in the German Bight in the future, allowing authorities to predict and mitigate any public health risks in advance. However, to ensure a rather low public health risk by potentially pathogenic *Vibrio* spp., it is not only important to determine the current occurrence and abundance of those organisms but also crucial to predict the extent and potential of pathogenic strains to bloom.

Currently, only travel-related diarrheal cases of toxigenic *Vibrio cholerae* infections are notifiable in European countries. Due to climate change, *Vibrio* occurrence and abundance,

and *Vibrio*-associated infections are expected to rise in coastal waters. Measures and actions of health authorities are urgently demanded to reduce the infection risk for the public. Especially informing physicians about novel symptoms and disease patterns as well as enforcing compulsory notifications of non-Cholera infections will increase the knowledge and awareness of infections caused by potentially pathogenic *Vibrio* species. This is a crucial step for the establishment of a surveillance system for *Vibrio* infections in Europe.

Supplementary material

Supplementary material to Chapter I

Table S1.1: Geographical information. Sampling stations with corresponding geographic coordinates, distances to coast and the salinity range over the entire sampling period.

Station	Latitude N	Longitude E	Distance to Coast [km]	Salinity Min – Max (Median)
I	54.152	7.892	61.35	31.3 – 33.4 (32.3)
II	54.102	7.987	53.18	30.0 – 33.3 (31.2)
III	54.050	8.083	44.88	29.8 – 33.2 (30.7)
IV	53.990	8.312	28.60	28.2 – 32.7 (29.7)
V	53.950	8.500	15.91	21.8 – 30.4 (24.8)
VI	53.900	8.680	4.55	14.2 – 24.2 (17.9)

Table S1.2: Spatial and temporal trends of environmental variable Differences of environmental parameters by the categories sampling month and sampling station are tested by Kruskal-Wallis one-way ANOVA (H) statistics followed by Dunn post-hoc tests for pairwise comparisons. Significant differences among categories are depicted by asterisk. *p<0.05, **p<0.01, ***p<0.001, df degrees of freedom. Significant different categories of the Dunn post-hoc test are listed.

	Kruskal Wallis one-way ANOVA [H]		**Pairwise multiple comparison Dunn *post hoc* test [p < 0.05]**	
Environmental variables	**Sampling site (df = 5)**	**Sampling month (df = 8)**	**Sampling site**	**Sampling month**
Temperature	1.64	66.05***	-	(Aug, Sept) vs. (Jan, May, Nov, Dec)
Salinity	57.98***	10.01	(VI) vs. (I – IV) (V) vs. (I, II) (IV) vs. (I)	-
Chlorophyll a	16.74**	30.47***	(V) vs. (I)	-
DO	0.35	57.81***	-	(Aug, Sept) vs. (Jan, May, Nov, Dec)
cDOM	59.79***	7.19	(VI) vs. (I – IV) (V) vs. (I, II) (IV) vs. (I)	-
Turbidity	61.91***	6.28	(VI) vs. (I – III) (V) vs. (I, II) (IV) vs. (I)	-
SiO_4	29.94***	29.85***	(VI) vs. (I – IV)	(June, July) vs. (Nov)
PO_4	31.46***	17,21**	(VI) vs. (I – III) (V) vs. (I, III)	(June) vs. (Nov)
NO_2	32.98***	20.02**	(VI) vs. (I – III) (V) vs. (I, III)	(July) vs. (Nov)
NO_3	42.76***	22.50**	(VI) vs. (I – IV) (V) vs. (I, II)	(May) vs. (Aug, Oct)
NH_4	11.07	35,52***	-	(Nov) vs. (May, July, Aug, Sept); (Dec) vs. (Aug, Sept)

Table S1.3: Collinearity between environmental parameters and *Vibrio* spp. abundances (MPN * L^{-1}). Spearman rank order correlation coefficients are given for each combination. Significant values ($p < 0.05$) are depicted in bold. (Vp) *V. parahaemolyticus*, (Vv) *V. vulnificus*, (Vc) *V. cholerae*

Spearman rank correlation coefficients

	Vv	**Vc**	**Temp**	**Sal**	**DO**	**cDOM**	**Chl a**	**Turb**	**SiO_4**	**PO_4**	**NO_2**	**NO_3**	**NH_4**
Vp	**0,67**	**0,35**	**0,56**	**-0,32**	**-0,44**	**0,30**	0,17	0,10	**0,33**	**0,43**	0,14	-0,08	0,13
Vv		0,21	**0,73**	-0,21	**-0,62**	0,16	0,22	-0,10	0,15	**0,26**	-0,14	**-0,25**	-0,19
Vc			**0,23**	**-0,41**	-0,03	**0,42**	0,15	**0,33**	**0,25**	**0,41**	**0,40**	**0,33**	**0,30**
Temp				**-0,24**	**-0,82**	0,17	0,54	-0,09	-0,10	0,04	**-0,25**	**-0,25**	**-0,35**
Sal					0,02	**-0,99**	**-0,54**	**-0,75**	**-0,48**	**-0,58**	**-0,58**	**-0,74**	**-0,23**
DO						0,05	-0,21	0,15	0,07	0,01	**0,27**	**0,31**	**0,31**
cDOM							**0,51**	**0,79**	**0,51**	**0,62**	**0,64**	**0,76**	**0,30**
Chl a								**0,30**	-0,10	0,05	0,11	0,22	-0,22
Turb									**0,47**	**0,55**	**0,66**	**0,80**	**0,32**
SiO_4										**0,80**	**0,66**	**0,41**	**0,59**
PO_4											**0,68**	**0,47**	**0,61**
NO_2												**0,71**	**0,69**
NO_3													**0,35**

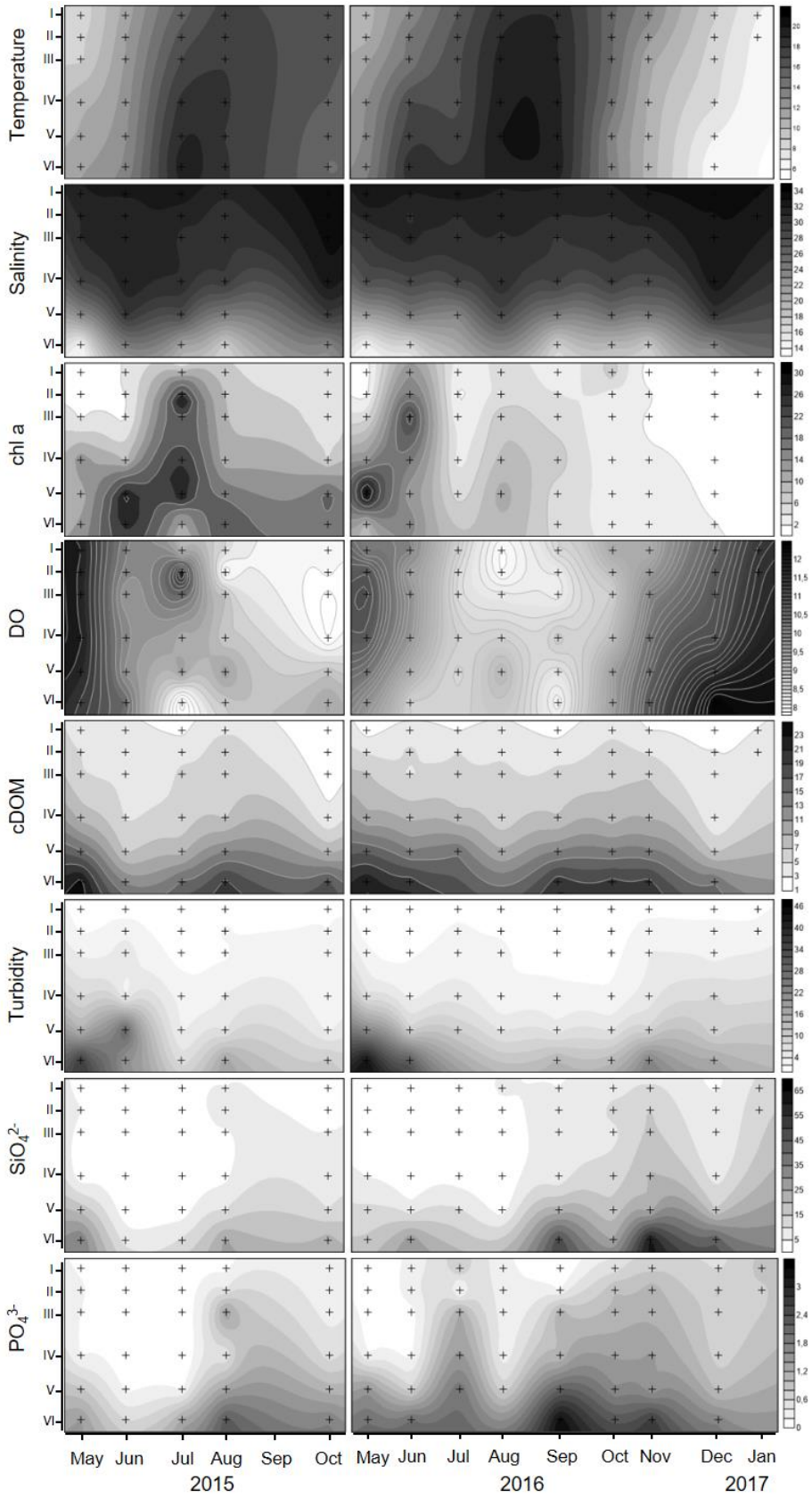

Figure S1.1: Environmental parameters recorded at each sampling site during the sampling campaigns. Depicted are sampling month and sampling site [I - VI]. Each cross represents one sampling point increasing colour intensity depicts increasing abundance. Smoothing by kriging

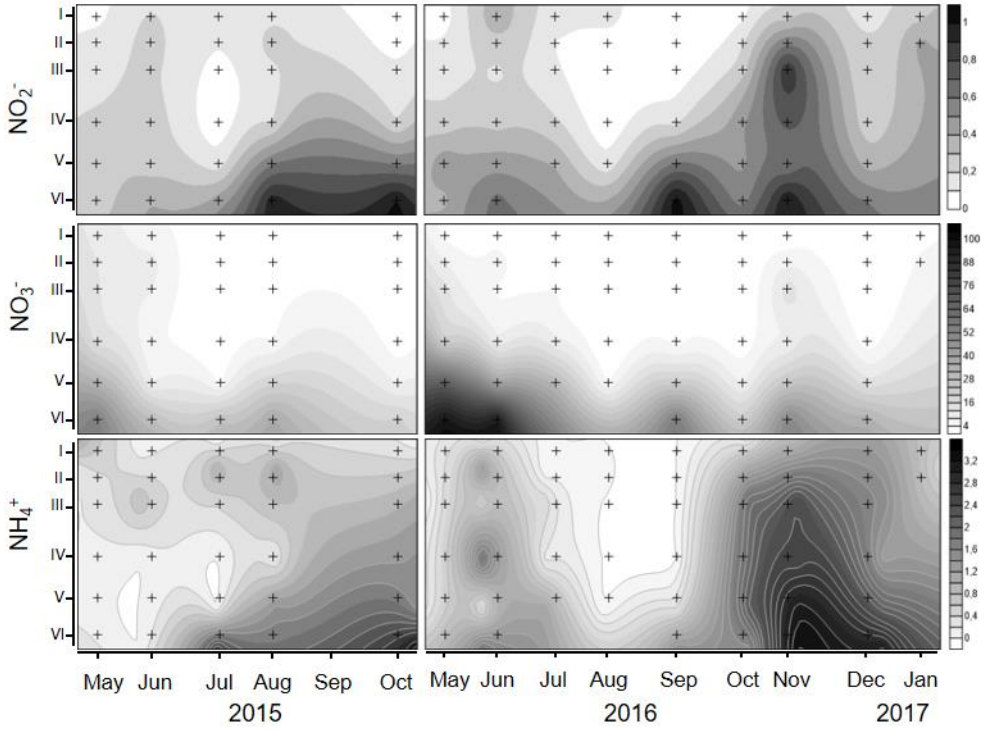

Figure S1.1 (continued): Environmental parameters recorded at each sampling site during the sampling campaigns. Depicted are sampling month and sampling site [I - VI]. Each cross represents one sampling point increasing colour intensity depicts increasing abundance. Smoothing by kriging

Table S1.4: Spatio-temporal occurrence of *Vibrio* spp. and of virulence-associated genes. Species-specific MPN-PCR results are given for each sampling point. Virulence-associated gene PCRs were conducted for positive species-specific PCR samples in at least duplicates (+ = positive PCR, - = negative PCR, blank = not analysed). (Vp) *V. parahaemolyticus*, (Vv) *V. vulnificus*, (Vc) *V. cholerae*

				Vp			Vv			Vc			Vp		Vv			Vc									
Sample #	Year	Month	Station	[MPN * L^{-1}]	lower 95 % - CI	upper 95 % - CI	[MPN * L^{-1}]	lower 95 % - CI	upper 95 % - CI	[MPN * L^{-1}]	lower 95 % - CI	upper 95 % - CI	*tdh*	*trh*	*nanA*	*manIIA*	PRXII^{+}	*O139*	*O1*	*ctx*	*tcpAET*	*chxA*	*TTSS*$^{\sigma}$	*hlyAET*$^{\delta}$	*VSP-II*	*rtxC*
1	2015	5	I	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														
2			II	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														
3			III	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														
4			IV	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														
5			V	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														
6			VI	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														
7		6	I	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														
8			II	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														
9			III	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														
10			IV	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														
11			V	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														
12			VI	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														
13		7	I	0,36	0,02	1,7	<0,3	0	0,95	< 0,3	0	0,95	-	-												
14			II	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														
15			III	0,36	0,02	1,7	<0,3	0	0,95	< 0,3	0	0,95	-	-												
16			IV	43	9	181	0,36	0,02	1,7	< 0,3	0	0,95	-	+	+	+	-									
17			V	2,1	0,5	4	0,36	0,02	1,7	< 0,3	0	0,95	-	-	+	+	+									
18			VI	2,3	0,5	9,4	0,36	0,02	1,7	9,3	1,8	36	-	-	+	+	+	-	-	-	-	-	+	+	-	+
19		8	I	110	40	350	74	13	200	< 0,3	0	0,95	-	-	+	+	+									
20			II	1,5	0,4	3,8	0,3	0,01	1	< 0,3	0	0,95	-	-	+	+	+									
21			III	93	18	360	21	5	40	< 0,3	0	0,95	-	-	+	+	+									
22			IV	4,3	0,9	18	3	0,1	10	0,74	0,13	2	-	-	+	+	+	-	-	-	-	+	-	+	+	+
23			V	2400	400	9900	4600	900	19800	2,3	0,5	9,4	-	-	+	+	+	-	-	-	-	-	+	+	-	+
24			VI	150	40	380	74	13	200	9,2	1,5	35	-	-	+	+	-	-	-	-	-	-	+	+	-	+
25		10	I	4,3	0,9	18,1	<0,3	0	0,95	< 0,3	0	0,95	-	-												
26			II	4,3	0,9	18,1	<0,3	0	0,95	< 0,3	0	0,95	-	-												
27			III	0,36	0,02	1,7	0,74	0,13	2	< 0,3	0	0,95	-	-	-	+	-									
28			IV	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														
29			V	24	4	99	<0,3	0	0,95	0,36	0,02	1,7	-	-				-	-	-	-	-	-	-	+	+
30			VI	43	9	181	<0,3	0	0,95	< 0,3	0	0,95	-	-												
31	2016	5	I	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														
32			II	<0,3	0	0,95	<0,3	0	0,95	0,3	0,01	1						-	-	-	-	-	-	+	-	-
33			III	<0,3	0	0,95	<0,3	0	0,95	0,36	0,02	1,7						-	-	-	-	-	-	+	-	+

34			IV	<0,3	0	0,95	<0,3	0	0,95	3	0,1	10						-	-	-	-	-	-	-	+	-
35			V	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														
36			VI	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														
37		6	I	0,3	0,01	1	<0,3	0	0,95	< 0,3	0	0,95	-	-												
38			II	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														
39			III	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														
40			IV	0,92	0,15	3,5	<0,3	0	0,95	4,3	0,9	18,1	-	-				-	-	-	-	+	+	+	+	+
41			V	1,5	0,4	3,8	<0,3	0	0,95	0,74	0,13	2	-	-	-	-	-	-	-	-	-	-	+	+	-	+
42			VI	<0,3	0	0,95	0,36	0,02	1,7	1,5	0,4	3,8			+	+	-	-	-	-	-	-	+	+	-	+
43		7	I	<0,3	0	1,95	4,3	0,9	18,1	< 0,3	0	0,95			+	+	-									
44			II	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														
45			III	4,3	0,9	18,1	1,5	0,4	3,8	< 0,3	0	0,95	-	+	+	+	+									
46			IV	9,3	1,8	36	<0,3	0	0,95	4,3	0,9	18,1	-	+				+	-	-	-	-	+	+	+	+
47			V	43	9	181	2,1	0,5	4	1,5	0,4	3,8	-	-	+	+	+	+	-	-	-	-	+	+	+	+
48		8	I	2,3	0,5	9,4	24	4	99	< 0,3	0	0,95	-	-	+	+	+									
49			II	4,3	0,9	18,1	150	40	380	< 0,3	0	0,95	-	-	+	+	+									
50			III	4,3	0,9	18,1	30	1	100	< 0,3	0	0,95	-	-	+	+	+									
51			IV	4,3	0,9	18,1	0,36	0,02	1,7	< 0,3	0	0,95	-	+	+	+	-									
52			V	74	13	200	240	40	990	0,74	0,13	2	-	+	+	+	+	-	-	-	-	-	-	+	-	+
53		9	I	4,3	0,9	18,1	4,3	0,9	18,1	< 0,3	0	0,95	-	-	+	+	+									
54			II	430	90	1810	43	9	181	< 0,3	0	0,95	-	-	+	+	+									
55			III	7,5	1,7	19,9	9,3	1,8	36	0,36	0,02	1,7	-	-	+	+	+	-	-	-	-	-	+	+	-	+
56			IV	24	4	99	2,3	0,5	9,4	0,36	0,02	1,7	-	-	+	+	-	-	-	-	-	-	-	+	-	+
57			V	150	40	380	74	13	200	24	4	99	-	+	+	+	+	+	-	-	-	-	+	+	+	+
58			VI	430	90	1810	1500	300	3800	3,6	0,2	17	-	+	+	+	+	-	-	-	-	-	+	+	-	+
59		10	I	2,1	0,5	4	1,5	0,4	3,8	< 0,3	0	0,95	-	-	+	+	+									
60			II	0,92	0,15	3,5	1,5	0,4	3,8	< 0,3	0	0,95	-	-	+	+	-									
61			III	4,3	0,9	18,1	1,5	0,4	3,8	< 0,3	0	0,95	-	-	+	+	+									
62			IV	15	4	38	0,74	0,13	2	0,92	0,15	3,5	-	-	+	+	+	-	-	-	-	-	-	+	-	+
63			V	43	9	181	3	0,1	10	0,36	0,02	1,7	-	+	+	+	+	-	-	-	-	-	-	-	-	+
64			VI	30	1	100	23	5	94	< 0,3	0	0,95	-	-	+	+	+									
65		11	I	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														
66			II	0,92	0,15	3,5	<0,3	0	0,95	< 0,3	0	0,95	-	-												
67			III	2,3	0,5	9,4	<0,3	0	0,95	0,92	0,15	3,5	-	-				-	-	-	-	-	-	-	+	+
68			IV	7,4	1,3	20	<0,3	0	0,95	0,92	0,15	3,5	-	-				-	-	-	-	-	-	-	-	+
69			V	24	4	99	<0,3	0	0,95	0,36	0,02	1,7	-	+				-	-	-	-	-	-	-	-	+
70			VI	74	13	200	<0,3	0	0,95	< 0,3	0	0,95	-	-												
71		12	I	<0,3	0	0,95	<0,3	0	0,95	0,36	0,02	1,7						-	-	-	-	-	-	+	+	+
72			II	<0,3	0	0,95	<0,3	0	0,95	0,92	0,15	3,5						-	-	-	-	+	-	+	-	+
73			III	0,74	0,13	2	<0,3	0	0,95	0,92	0,15	3,5	-	-				-	-	-	-	+	-	+	-	+
74			IV	1,1	0,4	3,5	<0,3	0	0,95	< 0,3	0	0,95	-	-												
75			V	0,92	0,15	3,5	<0,3	0	0,95	< 0,3	0	0,95	-	+												
76			VI	0,3	0,01	1	<0,3	0	0,95	< 0,3	0	0,95	-	-												
77	201	1	I	0,36	0,02	1,7	<0,3	0	0,95	< 0,3	0	0,95	-	-												

78			**II**	<0,3	0	0,95	<0,3	0	0,95	< 0,3	0	0,95														

[a] TTSS was targeted with four different primer pairs (*vcsC2*, *vcsN2*, *vspD* and *vcsV2*) and interpreted as present, when all four genes were present

[b] Absence of VSP-II is demonstrated by PCRs of the flanking region of the island. No visible PCR product was interpreted as VSP-II presence.

Table S1.5: Mann Whitney U Test on environmental dependence of the presence and absence of virulence-associated genes. Given are the U values and significance is depicted with asterisk (*p< 0.05, **p<0.01, ***p<0.001). (Vp) *V. parahaemolyticus*, (Vv) *V. vulnificus*, (Vc) *V. cholerae.* PRXII$^+$ stands for the presence of the gene *VVA1636/37*. TTSS was targeted with four different primer pairs (*vcsC2*, *vcsN2*, *vspD* and *vcsV2*) and interpreted as present, when all four genes were present. Absence of VSP-II is demonstrated by PCRs of the flanking region of the island. No visible PCR product was interpreted as VSP-II presence.

Mann Whitney U Test [U]

	Vp	**Vv**	**Vc**					
Environmental Variables	***trh***	**PRXII$^+$**	***O139***	***TTSS***	***hlyAET***	***VSP-II***	***rtxC***	***chxA***
Temperature	153	89	14	25**	28	68	10	27
Salinity	134	78	9	21***	59	61	17	20
Chlorophyll a	168	70	21	42*	54	72	23	37
Turbidity	118	84	15	33**	51	69	17	31
SiO_4	182	83	13	56	35	55	6	22
PO_4	138	85.5	5	36*	54	64	2*	19
NO_2	185	87.5	17	53	30	74	10	31
NO_3	133	84	10	24**	57	75	21	23
NH_4	191.5	85	20	82	23*	75	10	33
MPN L^{-1}	111*	51	8	21***	42	55	18	37

Table S1.6: *V. vulnificus* virulence profiles (VvP). Number of positive samples, percentage of all samples (N = 78) and percentage of *V. vulnificus* (Vv) positive samples (N = 31). PRXII$^+$ stands for the presence of the gene *VVA1636/37.*

Profile #	Virulence profile	No. of samples	% of all	% of Vv
VvP-1	*vvhA$^+$ manIIA$^+$*	1	1	3
VvP-2	*vvhA$^+$ manIIA$^+$ nanA$^+$*	7	9	23
VvP-3	*vvhA$^+$ manIIA$^+$ nanA$^+$* PRXII$^+$	23	30	74

Table S1.7: *V. cholerae* virulence profiles (VcP). Number of positive samples, percentage of all samples (N = 78) and percentage of *V. cholerae* (Vc) positive samples (N = 26). TTSS was targeted with four different primer pairs (vcsC2, vcsN2, vspD and vcsV2) and interpreted as present, when all four genes were present. Absence of VSP-II is demonstrated by PCRs of the flanking region of the island. No visible PCR product was interpreted as VSP-II presence.

Profile #	Virulence profile	No. of samples	% of all	% of Vc
VcP-1	*Vc toxR$^+$ rtxC$^+$*	3	4	12
VcP-2	*Vc toxR$^+$ hlyAET$^+$*	1	1	4
VcP-3	*Vc toxR$^+$ VSP-II$^+$*	1	1	4
VcP-4	*Vc toxR$^+$ rtxC$^+$ VSP-II$^+$*	2	3	8
VcP-5	*Vc toxR$^+$ rtxC$^+$ hlyAET$^+$*	4	5	15
VcP-6	*Vc toxR$^+$ rtxC$^+$ hlyAET$^+$ VSP-II$^+$*	1	1	4
VcP-7	*Vc toxR$^+$ rtxC$^+$ hlyAET$^+$ chxA$^+$*	2	3	8
VcP-8	*Vc toxR$^+$ rtxC$^+$ hlyAET$^+$ TTSS$^+$*	7	9	27
VcP-9	*Vc toxR$^+$ rtxC$^+$ hlyAET$^+$ VSP-II$^+$ chxA$^+$*	1	1	4
VcP-10	*Vc toxR$^+$ rtxC$^+$ hlyAET$^+$ VSP-II$^+$ TTSS$^+$*	1	1	4
VcP-11	*Vc toxR$^+$ rtxC$^+$ hlyAET$^+$ VSP-II$^+$ TTSS$^+$ chxA$^+$*	1	1	4
VcP-12	*Vc toxR$^+$ rtxC$^+$ hlyAET$^+$ VSP-II$^+$ TTSS$^+$ O139$^+$*	2	4	12

Supplementary material to Chapter II

Table S2.1: Growth media used for high-nutrient growth assays.

Growth medium #	Conductivity [mS/cm]	Salinity
1	1,1	0,7
2	2,9	2,6
3	4,9	4,8
4	6,8	6,8
5	8,8	9,0
6	10,5	10,8
7	12,6	13,1
8	14,5	15,1
9	16,2	16,9
10	18,5	19,4
11	20,4	21,5
12	22,3	23,5
13	24,5	25,9
14	26	27,5
15	28,2	29,8
16	30,3	32,1
17	32,3	34,2
18	34,1	36,2
19	36,2	38,4

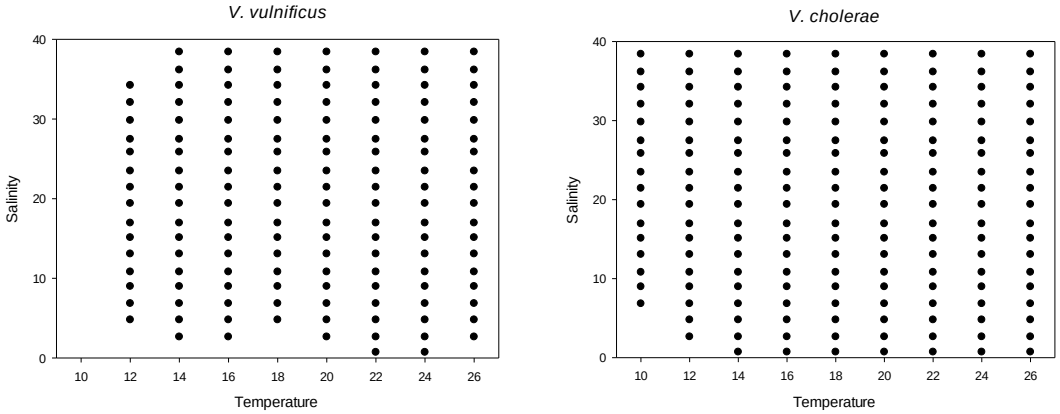

Figure S2.1: Ecological niche experiments of *V. cholerae* and *V. vulnificus* showing growth at different temperature and salinity conditions are depicted with dots. Conditions where no growth was detected are empty.

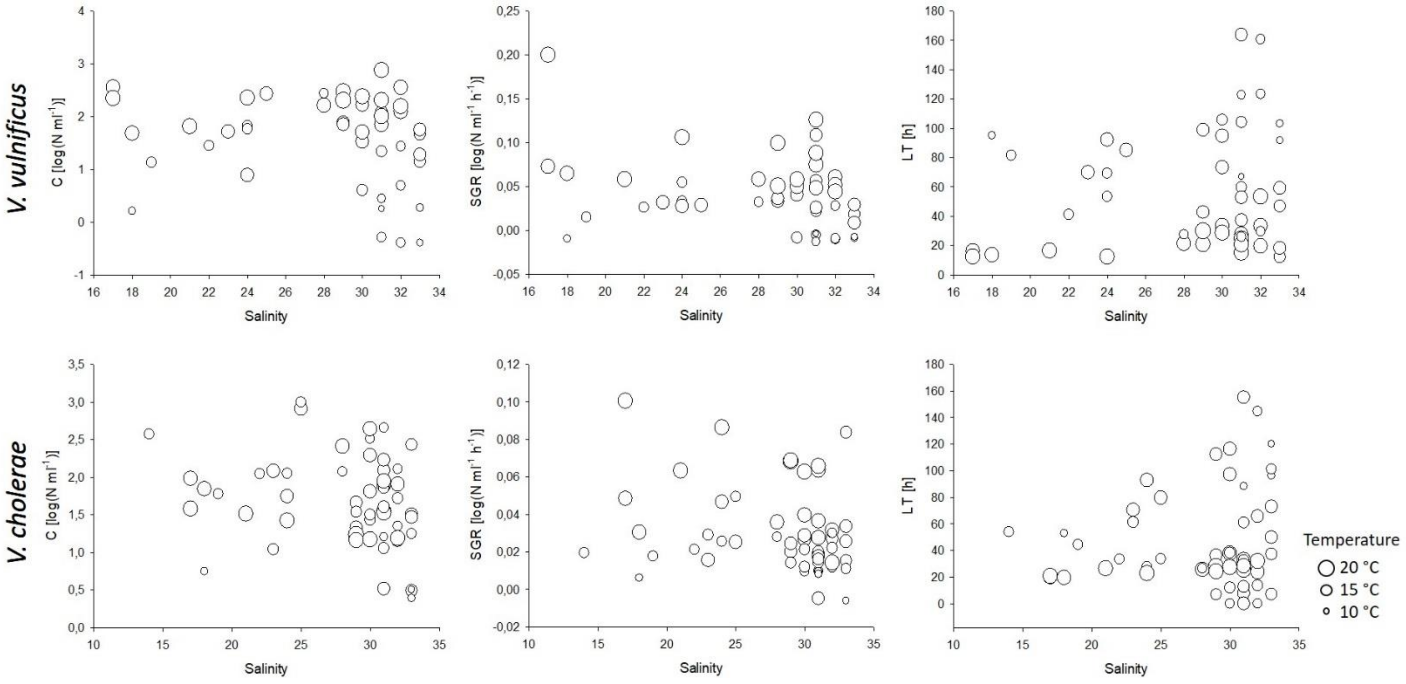

Figure S2.2: Growth parameters of *V. cholerae* and *V. vulnificus* under low-nutrient conditions changing with salinity. Temperature is depicted in the bubble size.

Table S2.2: Mann Whitney Rank Sum Test (U) of Niche and PGP growth parameters for *V. vulnificus* and *V. cholerae*

		p	significant	U
V. vulnificus	C	<0.0001	***	272
	SGR	0.0013	**	1161
	LT	<0.0001	***	611
V. cholerae	C	<0.0001	***	71
	SGR	<0.0001	***	1478
	LT	0.0124	*	1961

Supplementary material to Chapter III

Table S3.1: Sampling stations with sampling dates and corresponding geographic coordinates of sampling sites.

HE409 2013				HE430 2014			
Station No.	**Sampling Date**	**Latitude N**	**Longitude E**	**Station No.**	**Sampling Date**	**Latitude N**	**Longitude E**
1	19.09.2013	54,0822	7,4608	**39**	31.07.2014	53,8252	7,7673
2	19.09.2013	53,9931	6,9928	**40**	31.07.2014	53,7947	7,3492
3	19.09.2013	53,8681	6,4367	**41**	01.08.2014	53,7475	6,9987
4	20.09.2013	53,7061	6,6381	**42**	01.08.2014	53,7177	6,6760
5	20.09.2013	53,4842	6,8097	**43**	01.08.2014	53,6513	6,3315
6	20.09.2013	53,3183	7,0392	**44**	02.08.2014	53,6130	6,1380
7	21.09.2013	53,8256	7,1300	**45**	02.08.2014	53,5530	5,5923
8	21.09.2013	53,8897	7,6250	**46**	02.08.2014	53,4747	5,1825
9	21.09.2013	53,6847	8,0892	**47**	03.08.2014	53,3033	4,8048
10	21.09.2013	53,5269	8,1800	**48**	03.08.2014	53,1422	4,6017
11	22.09.2013	53,5539	8,5547	**49**	03.08.2014	52,9177	4,4325
12	22.09.2013	53,7222	8,2764	**50**	04.08.2014	52,4260	4,3475
13	22.09.2013	53,8344	8,1394	**51**	04.08.2014	52,1702	4,0132
14	22.09.2013	54,0000	8,0264	**52**	04.08.2014	51,8667	3,6258
15	22.09.2013	54,1489	7,8858	**53**	05.08.2014	51,5395	3,1822
16	23.09.2013	54,3328	7,7178	**54**	05.08.2014	51,2847	2,5150
17	23.09.2013	54,6958	7,9758	**55**	05.08.2014	51,0777	1,9037
18	23.09.2013	54,4928	8,0947	**56**	06.08.2014	50,4965	1,1654
19	23.09.2013	54,2667	8,2956	**57**	06.08.2014	51,5836	2,4426
20	24.09.2013	54,1056	8,3936	**58**	07.08.2014	52,1503	2,8428
21	24.09.2013	53,9439	8,6719	**59**	07.08.2014	52,9783	3,2288
22	24.09.2013	53,8819	9,0658	**60**	08.08.2014	53,9062	3,1847
23	25.09.2013	54,3433	10,1742	**61**	08.08.2014	54,8117	3,3883
24	25.09.2013	54,6528	10,1697	**62**	09.08.2014	55,8355	3,5624
25	25.09.2013	54,7356	10,1739	**Helgoland drift line 2013**			
26	25.09.2013	54,8333	9,8628	**63**	01.08.2013	54,2875	7,9000
27	26.09.2013	54,5550	10,8672				
28	26.09.2013	54,5822	11,0358				
29	26.09.2013	54,3889	11,5358				
30	26.09.2013	54,0842	11,1842				
31	27.09.2013	54,2861	12,0853				
32	27.09.2013	54,6108	12,3831				
33	27.09.2013	54,8261	13,0408				
34	27.09.2013	54,8333	13,7525				
35	28.09.2013	54,7058	14,3600				
36	28.09.2013	54,5117	14,2575				
37	28.09.2013	54,2375	14,2839				
38	28.09.2013	53,9975	14,2272				

Table S3.2: Water volume which passed through the Neuston net (300 µm), determined by the use of a mechanical flowmeter.

HE409 2013					HE430 2014				
Station No.	**Start Flow**	**End Flow**	**Liter**	**m³**	**Station No.**	**Start Flow**	**End Flow**	**Liter**	**m³**
1	49649	57284	68715	68,72	**39**	92509	100113	68436	68,44
2	62367	67824	49113	49,11	**40**	4196	14306	90990	90,99
3	72922	77213	38619	38,62	**41**	18813	27785	80748	80,75
4	83701	91832	73179	73,18	**42**	32919	44963	108396	108,40
5	96327	101807	49320	49,32	**43**	49906	57772	70794	70,79
6	3433	7619	37674	37,67	**44**	60029	69737	87372	87,37
7	9638	16657	63171	63,17	**45**	73637	79552	53235	53,24
8	19047	27835	79092	79,09	**46**	82292	90188	71064	71,06
9	31024	43069	108405	108,41	**47**	94068	106096	108252	108,25
10	46675	54364	69201	69,20	**48**	10651	21495	97596	97,60
11	57752	67578	88434	88,43	**49**	25453	36755	101718	101,72
12	68990	77696	78354	78,35	**50**	39892	48927	81315	81,32
13	80181	86948	60903	60,90	**51**	50817	60723	89154	89,15
14	89189	97154	71685	71,69	**52**	63436	75348	107208	107,21
15	1698	7924	56034	56,03	**53**	77074	88854	106020	106,02
16	14722	21229	58563	58,56	**54**	93352	105088	105624	105,62
17	28134	36277	73287	73,29	**55**	7596	18520	98316	98,32
18	39255	47167	71208	71,21	**56**	22866	34363	103473	103,47
19	50437	57539	63918	63,92	**57**	38202	50200	107982	107,98
20	61413	71174	87849	87,85	**58**	53857	68068	127899	127,90
21	73193	84028	97515	97,52	**59**	72092	86125	126297	126,30
22	85496	95390	89046	89,05	**60**	91829	106432	131427	131,43
23	119	5203	45756	45,76	**61**	11632	23306	105066	105,07
24	6415	15374	80631	80,63	**62**	28821	40817	107964	107,96
25	19624	27148	67716	67,72					
26	31600	38955	66195	66,20					
27	42023	51727	87336	87,34					
28	56054	60557	40527	40,53					
29	66420	74847	75843	75,84					
30	79712	82547	25515	25,52					
31	84794	95416	95598	95,60					
32	928	10663	87615	87,62					
33	16057	24210	73377	73,38					
34	27942	35303	66249	66,25					
35	40476	47486	63090	63,09					
36	53208	60592	66456	66,46					
37	66177	75699	85698	85,70					
38	82645	89783	64242	64,24					

Table S3.3: Environmental parameters. Temperatures and salinities recorded at each station.

HE 409 2013			HE 430 2014		
Station No.	**°C**	**PSU**	**Station No.**	**°C**	**PSU**
1	16,9	32,31	**39**	19,70	32,6
2	17,25	32,57	**40**	20,43	32,32
3	17,14	32,85	**41**	21,13	32,18
4	15,77	31,86	**42**	21,65	31,66
5	14,90	30,36	**43**	20,73	32,29
6	15,07	25,14	**44**	20,70	32,56
7	16,72	31,48	**45**	20,95	33,08
8	16,83	31,50	**46**	20,50	33,18
9	15,15	30,68	**47**	20,08	32,77
10	15,17	31,19	**48**	19,85	33,00
11	15,70	14,23	**49**	19,96	34,00
12	15,10	25,30	**50**	20,54	30,96
13	15,74	30,82	**51**	20,04	31,62
14	16,88	32,16	**52**	20,45	32,48
15	16,64	32,21	**53**	20,71	32,40
16	16,19	31,74	**54**	19,56	34,16
17	16,23	29,45	**55**	18,83	34,33
18	16,08	29,64	**56**	18,34	33,77
19	15,82	28,47	**57**	18,79	34,46
20	15,38	27,64	**58**	18,38	33,53
21	15,66	16,89	**59**	18,89	33,51
22	16,85	3,03	**60**	17,73	34,00
23	15,24	15,93	**61**	19,07	34,00
24	15,27	15,73	**62**	18,70	34,20
25	15,41	15,5	**Helgoland drift line 2013**		
26	15,35	16,61	**63**	16,60	30,23
27	14,93	16,8			
28	14,79	15,27			
29	14,95	12,99			
30	14,84	12,64			
31	14,07	11,65			
32	14,56	8,75			
33	15,11	7,59			
34	14,52	7,43			
35	15,25	7,26			
36	14,67	7,27			
37	14,85	7,06			
38	14,46	5,67			

Table S3.4: Occurrence of visible particles collected during the North and Baltic Sea cruises and on Helgoland beach. Stations and corresponding collected particle samples, identity of the material and the corresponding HIT-Score of ATR-FT IR analysis are display here (Hit-Scores of ≥700 were accepted. Any matches with quality index <700 were individually inspected and interpreted based on the closeness of their absorption frequencies to those of chemical bonds in the known polymers, N.i. = Not identified).

HE 409 2013				HE 430 2014			
Station No.	**Sample No.**	**Material**	**ATR-FT IR HIT-Score**	**Station No.**	**Sample No.**	**Material**	**ATR-FT IR HIT-Score**
1	1P1	Varnish	646	**39**	1P1	Acrylnitril-Butadien-Styrol	917
1	1P2	Keratin	185	**39**	1P2	Polystyrene	994
1	1P3	Polyethylene	794	**39**	1P3	Polystyrene	915
2	2P1	Polystyrene	996	**39**	1P4	Varnish	653
2	2P2	Polyethylene	998	**40**	2P1	Polyethylene	795
2	2P3	Polypropylene	903	**40**	2P2	Polyethylene	995
2	2P4	Polystyrene	997	**40**	2P3	Polyethylene	993
2	2P5	Polystyrene	840	**40**	2P4	Polypropylene	920
2	2P6	N.i.	382	**40**	2P5	Polyethylene	784
3	3P1	Polyvinylalcohol	329	**41**	3P1	Polyethylene	817
3	3P2	Varnish	504	**41**	3P2	Polyethylene	997
3	3P3	Polyethylene	298	**41**	3P3	Polyethylene	998
3	3P4	Polypropylene	747	**41**	3P4	Polyethylene	993
3	3P5	Polypropylene	795	**41**	3P5	Polyethylene	990
3	3P6	Polystyrene	901	**41**	3P6	Polyethylene	993
4	4P1	Polyethylene	986	**41**	3P7	Polyethylene	846
4	4P2	Ethylen-vinylalcohol	968	**41**	3P8	Polypropylene	884
4	4P3	Polyethylene	997	**42**	4P1	Polypropylene	696
4	4P4	Polypropylene	933	**42**	4P2	Varnish	560
4	4P5	Ethylen-vinylalcohol	947	**43**	5P1	Polyethylene	989
4	4P6	Polyethylene	997	**43**	5P2	Polyethylene	797
4	4P7	Polyethylene	997	**43**	5P3	Polypropylene	812
5	5P1	Polypropylene	755	**47**	9P2	Polyethylene	398
5	5P2	Polyethylene	790	**49**	11P1	Polystyrene	986
5	5P3	Polyethylene	695	**49**	11P2	Polyethylene	796
6	6P1	Chitin	542	**49**	11P3	Polyethylene	944
6	6P2	Chitin	723	**49**	11P4	Chitin	608
6	6P3	Polyethylene	987	**50**	12P1	Polyethylene	823
6	6P4	Polypropylene	877	**52**	14P1	Polypropylene	854
6	6P5	Polypropylene	563	**52**	14P2	Polyethylene	819
6	6P6	Chitin	612	**52**	14P3	Polyethylene	707
7	7P1	Polypropylene	871	**52**	14P4	Polyethylene	796
7	7P2	Polyethylene	695	**53**	15P1	Polypropylene	852
7	7P3	Polyethylene	837	**55**	17P1	Polyethylene	644
7	7P4	Polyethylene	723	**55**	17P2	Polyethylene	609
8	8P1	Polyethylene	838	**56**	18P1	Polyethylene	786
8	8P2	Polyethylene	838	**56**	18P2	Polypropylene	795
8	8P3	Polyethylene	996	**56**	18P3	Polyethylene	701
8	8P4	Polyethylene	998	**56**	18P4	Polyethylene	529

8	8P5	Polyamide	487	**56**	18P5	Stearic Acid	291
9	9P1	Ethylen-vinylalcohol	645	**56**	18P6	Polypropylene	450
9	9P2	Polypropylene	852	**56**	18P7	Polypropylene	688
9	9P3	Polyethylene	564	**56**	18P8	Polyethylene	466
9	9P4	Polypropylene	841	**56**	18P9	Polyethylene	891
9	9P5	Polyethylene	778	**56**	18P10	Polyethylene	820
10	10P1	Polyethylene	988	**56**	18P11	Chitin	447
11	11P1	Polyethylene	796	**56**	18P12	Polypropylene	848
13	13P1	Polyethylene	837	**56**	18P13	Polystyrene	984
14	14P1	Polyethylene	836	**57**	19P1	Polypropylene	562
15	15P1	Polyethylene	820	**57**	19P2	Polyethylene	703
15	15P2	Polyethylene	839	**57**	19P3	Polyethylene	663
15	15P3	Polyethylene	837	**57**	19P4	Polyethylene	406
15	15P4	Polyethylene	725	**57**	19P5	Polyethylene	387
15	15P5	Polyethylene	996	**58**	20P1	N.i.	
15	15P6	Polyethylene	726	**58**	20P2	N.i.	
15	15P7	Polyethylene	726	**58**	20P3	N.i.	
16	16P1	Polyethylene	692	**58**	20P4	N.i.	
16	16P2	Polyethylene	993	**58**	20P5	N.i.	
16	16P3	Polyethylene	516	**58**	20P6	N.i.	
16	16P4	Polypropylene	890	**58**	20P7	N.i.	
16	16P5	Polyethylene	837	**58**	20P8	N.i.	
16	16P6	Polyethylene	725	**58**	20P9	N.i.	
16	16P7	Polyethylene	815	**58**	20P10	N.i.	
16	16P8	Chitin	449	**59**	21P1	N.i.	
17	17P1	Polypropylene	695	**59**	21P2	N.i.	
17	17P2	Polyethylene	985	**59**	21P3	N.i.	
17	17P3	Polyethylene	997	**59**	21P4	N.i.	
17	17P4	Polyethylene	541	**59**	21P5	N.i.	
17	17P5	Polypropylen	701	**60**	22P1	N.i.	
17	17P6	Polyethylene	800	**60**	22P2	N.i.	
17	17P7	Polypropylen	422	**60**	22P3	N.i.	
17	17P8	Polyethylene	995	**60**	22P4	N.i.	
17	17P9	Polyethylene	993	**61**	23P1	N.i.	
17	17P10	Polyethylene	837	**61**	23P2	N.i.	
18	18P1	Polyethylene	574	**61**	23P3	N.i.	
18	18P2	Polyethylene	641	**61**	23P4	N.i.	
18	18P3	Polyethylene	726	**61**	23P5	N.i.	
18	18P4	Polyethylene	839	**61**	23P6	N.i.	
18	18P5	Polyethylene	469	**61**	23P7	N.i.	
18	18P6	Polyethylene	994	**61**	23P8	N.i.	
19	19P1	Polystyrene	595	**61**	23P9	N.i.	
21	21P1	Polyethylene	568	**61**	23P10	N.i.	
21	21P2	Polyethylene	564	**Helgoland drift line 2013**			
21	21P3	Polystyrene	697	**63**	63P1	Polyethylene	794
21	21P4	Polyethylene	751	**63**	63P2	Polyvinylchloride	494
22	22P1	Polypropylen	669	**63**	63P3	Polyethylene	994

22	22P2	Polypropylen	909
22	22P3	Polypropylen	652
23	23P1	Polystyrene	669
23	23P2	Polyethylene	994
26	26P1	Keratin	550
29	29P1	Chitin	529
30	30P1	Polypropylene	994
32	32P1	Polyethylene	724
32	32P2	Keratin	364
33	33P1	Keratin	285
35	35P1	Keratin	654
35	35P2	Keratin	460
36	36P1	Keratin	597
37	37P1	Polyethylene	658
37	37P2	Polyethylene	724
37	37P3	Polyethylene	726
38	38P1	N.i.	299
63	63P4	Polypropylene	990
63	63P5	Polyethylene	793
63	63P6	Polypropylene	929
63	63P7	Polypropylene	605
63	63P8	Polyamide	917
63	63P9	Polyethylene	903
63	63P10	Polyurethane	311
63	63P11	Polyethylene	773
63	63P12	Varnish	175
63	63P13	Polyvinylchloride	478
63	63P14	Polystyrene	994
63	63P15	Polyethylene	999

Table S3.5: MALDI-TOF *Vibrio* identification results & species-specific and virulence-associated-gene PCR results (+ = positive, - = negative) of *V. parahaemolyticus* obtained from microplastic samples. Meaning of MALDI HIT-Score: 2.300-3.000 highly probable species identification, 2.000-2.299 secure genus – probable species identification, 1.700-1.999 probable genus identification, 0.000-1.699 not reliable identification.

Station No.	Particle No.	Polymer type	Isolate label	Species identification	MALDI HIT-Score	*toxR*	*tdh*	*trh*
63	63P1	PE	1A	*V. parahaemolyticus*	2,59	+	-	-
63	63P4	PP	4B	*V. parahaemolyticus*	2,61	+	-	-
63	63P6	PP	6A	*V. parahaemolyticus*	2,58	+	-	-
63	63P9	PE	9A	*V. parahaemolyticus*	2,62	+	-	-
5	5P2	PE	VN-4252	*V. parahaemolyticus*	2,40	+	-	-
5	5P2	PE	VN-4253	*V. parahaemolyticus*	2,55	+	-	-
9	9P3	PE	VN-4225	*V. parahaemolyticus*	2,62	+	-	-
11	11P1	PE	VN-4229	*V. fluvialis*	2,53			
21	21P2	PE	VN-4237	*V. parahaemolyticus*	2,54	+	-	-
30	30P1	PP	VN-4239	*V. parahaemolyticus*	2,62	+	-	-
30	30P1	PP	VN-4240	*V. fluvialis*	2,55			
39	39P3	PS	VN-3234	*V. parahaemolyticus*	2,47	+	-	-
41	41P1	PE	VN-3228	*V. parahaemolyticus*	2,22	+	-	-
41	41P3	PE	VN-3231	*V. parahaemolyticus*	2,38	+	-	-
41	41P4	PE	VN-3225	*V. parahaemolyticus*	2,42	+	-	-
41	41P6	PE	VN-3232	*V. alginolyticus*	2,35			
55	55P2	PE	VN-3227	*V.spp*	1,91			
55	55P2	PE	VN-3229	*V.spp*	1,96			
58	58P9	NI	VN-3224	*V. fluvialis*	2,57			
58	58P10	NI	VN-3226	*V. fluvialis*	2,44			

58	58P7	NI	VN-3233	*V. fluvialis*	2,53			
59	59P4	NI	VN-3230	*V. fluvialis*	2,28			

Table S3.6: MALDI-TOF *Vibrio* identification results & species-specific and virulence-associated-gene PCR results (+ = positive, - = negative) of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* obtained from water samples. Meaning of MALDI HIT-Score: 2.300-3.000 highly probable species identification, 2.000-2.299 secure genus – probable species identification, 1.700-1.999 probable genus identification, 0.000-1.699 not reliable identification.

Station No.	Isolate label	Species identification	MALDI HIT-Score	*toxR*	*tdh*	*trh*	*O1*	*O139*	*ctxA*
1	VN-4208	*V. diazotrophicus*	2,47						
1	VN-4209	*V. diazotrophicus*	2,54						
2	VN-4210	*V. vulnificus*	2,62	+					
2	VN-4211	*V. vulnificus*	2,60	+					
3	VN-4212	*V. parahaemolyticus*	2,61	+	+	-			
3	VN-4213	*V. parahaemolyticus*	2,64	+	-	-			
4	VN-4214	*V. mimicus*	2,56						
4	VN-4215	*V. mimicus*	2,57						
5	VN-4216	*V. cholerae*	2,64	+			-	-	-
5	VN-4217	*V. parahaemolyticus*	2,67	+	-	-			
5	VN-4218	*V. parahaemolyticus*	2,62	+	-	-			
6	VN-4219	*V. cholerae*	2,52	+			-	-	-
6	VN-4231	*V. cholerae*	2,52	+			-	-	-
6	VN-4220	*V. parahaemolyticus*	2,67	+	-	-			
8	VN-4221	*V. vulnificus*	2,62	+					
8	VN-4222	*V. fluvialis*	2,50						
9	VN-4223	*V. cholerae*	2,62	+			-	-	-
9	VN-4224	*V. parahaemolyticus*	2,64	+	-	-			
10	VN-4226	*V. cholerae*	2,65	+			-	-	-
10	VN-4227	*V. parahaemolyticus*	2,55	+	-	-			
11	VN-4228	*V. parahaemolyticus*	2,56	+	-	-			
12	VN-4230	*V. parahaemolyticus*	2,61	+	-	-			
12	VN-4254	*V. mimicus*	2,41						
12	VN-4255	*V. parahaemolyticus*	2,70	+	-	-			
12	VN-4261	*V. cholerae*	2,63	+			-	-	-
13	VN-4262	*V. parahaemolyticus*	2,67	+	-	-			
13	VN-4263	*V. parahaemolyticus*	2,72	+	-	-			
16	VN-4256	*V. vulnificus*	2,52	+					
16	VN-4243	*V. diazotrophicus*	2,43						
17	VN-4257	*V. fluvialis*	2,53						
17	VN-4264	*V.* mechnikovii	2,26						
18	VN-4258	*V. fluvialis*	2,57						
19	VN-4265	*V. parahaemolyticus*	2,68	+	-	-			
19	VN-4259	*V. parahaemolyticus*	2,63	+	-	-			
20	VN-4232	*V. parahaemolyticus*	2,67	+	-	-			

21	VN-4233	*V. cholerae*	2,58	+			-	-	-
21	VN-4234	*V. mimicus*	2,48						
21	VN-4235	*V. parahaemolyticus*	2,46	+					
21	VN-4236	*V. parahaemolyticus*	2,66	+	-	-			
25	VN-4238	*V. diazotrophicus*	2,45						
31	VN-4241	*V. cholerae*	2,65	+			-	-	-
32	VN-4242	*V. fluvialis*	2,49						
35	VN-4248	*V. diazotrophicus*	2,61						
36	VN-4244	*V. vulnificus*	2,48	+					
36	VN-4245	*V. vulnificus*	2,43	+					
36	VN-4246	*V. vulnificus*	2,53	+					
36	VN-4247	*V. cholerae*	2,48	+			-	-	-
37	VN-4249	*V. vulnificus*	2,59	+					
38	VN-4250	*V. cholerae*	2,64	+			-	-	-
38	VN-4251	*V. cholerae*	2,66	+			-	-	-
39	VN-3253	*V. fluvialis*	2,43						
39	VN-3257	*V. parahaemolyticus*	2,49	+	-	-			
39	VN-3280	*V. vulnificus*	2,60	+					
40	VN-3268	*V. parahaemolyticus*	2,41	+	-	-			
41	VN-3265	*V. parahaemolyticus*	2,60	+	-	-			
42	VN-3255	*V. parahaemolyticus*	2,34	+	-	-			
42	VN-3266	*V. parahaemolyticus*	2,24	+	-	-			
42	VN-3245	*V.spp*	1,78						
42	VN-3250	*V. spp.*	2,21						
42	VN-3275	*V. parahaemolyticus*	2,60	+	-	-			
43	VN-3262	*vulnificus*	2,34	+					
43	VN-3236	*V.spp*	1,66						
43	VN-3251	*V. parahaemolyticus*	2,49	+	-	-			
43	VN-3252	*V. vulnificus*	2,48	+					
43	VN-3269	*V. fluvialis*	2,43						
44	VN-3244	*V. parahaemolyticus*	2,47	+	-	-			
45	VN-3282	*V. parahaemolyticus*	2,66	+	-	-			
45	VN-3271	*V. vulnificus*	2,48	+					
47	VN-3261	*V. parahaemolyticus*	2,41	+	-	-			
48	VN-3277	*V. vulnificus*	2,33	+					
48	VN-3278	*V. parahaemolyticus*	2,53	+	-	-			
48	VN-3273	*V. parahaemolyticus*	2,60	+	-	-			
48	VN-3256	*V. mimicus*	2,63						
49	VN-3270	*V. fluvialis*	2,37						
49	VN-3260	*V. fluvialis*	2,41						
49	VN-3239	*V. fluvialis*	2,37						
51	VN-3284	*V. fluvialis*	2,39						
51	VN-3285	*V. parahaemolyticus*	2,60	+	-	-			
51	VN-3286	*V. fluvialis*	2,45						
51	VN-3272	*V. parahaemolyticus*	2,50	+	-	-			
51	VN-3263	*V. parahaemolyticus*	2,32	+	-	-			
51	VN-3274	*V. parahaemolyticus*	2,45	+	-	-			

51	VN-3259	*V. vulnificus*	2,41	+					
51	VN-3276	*V. vulnificus*	2,55	+					
51	VN-3254	*V. parahaemolyticus*	2,33	+	-	-			
51	VN-3249	*V. parahaemolyticus*	2,38	+	-	-			
51	VN-3279	*V. vulnificus*	2,64	+					
51	VN-3246	*V. fluvialis*	2,45						
51	VN-3241	*V. mimicus*	2,51						
51	VN-3242	*V. fluvialis*	2,38						
51	VN-3237	*V. mimicus*	2,52						
52	VN-3240	*V. parahaemolyticus*	2,44	+	-	-			
52	VN-3247	*V. parahaemolyticus*	2,64	+	-	-			
52	VN-3264	*V. vulnificus*	2,55	+					
52	VN-3287	*V. fluvialis*	2,60						
53	VN-3258	*V. parahaemolyticus*	2,69	+	-	-			
58	VN-3235	*V. fluvialis*	2,42						
58	VN-3281	*V. fluvialis*	2,53						
58	VN-3288	*V. fluvialis*	2,58						
62	VN-3267	*V. fluvialis*	2,58						
62	VN-3238	*V. fluvialis*	2,50						
62	VN-3283	*V. fluvialis*	2,43						
62	VN-3243	*V. fluvialis*	2,52						
62	VN-3248	*V. fluvialis*	2,42						

Supplementary material to General discussion

Table S4.1: Infection case reports in northern Europe (1973 – 2014) caused by *Vibrio* spp. (adopted from (Vezzulli et al., 2016))

Year	Country	No. of cases	*Vibrio* species	Reference
1973	United Kingdom	12	*V. parahaemolyticus*	Hooper et al. (1974)
1977	Belgium	1	*V. vulnificus*	Mertens et al. (1979)
1991	Denmark	1	*V. vulnificus*	Andersen (1991)
1992	Netherlands	6	*V. vulnificus*, *V. parahaemolyticus*, *Vibrio alginolyticus*	Veenstra et al. (1993)
1994	Germany, Sweden, Denmark, Finland	21	*V. cholerae* (non-O1/O139), *V. vulnificus*, *V. alginolyticus*	Baker-Austin et al. (2013)
1995	Denmark, Finland, Estonia	8	*V. cholerae* (non-O1/O139), *V. vulnificus*	Baker-Austin et al. (2013)
1996	Denmark, Finland	2	*V. cholerae* (non-O1/O139)	Baker-Austin et al. (2013)
1997	Sweden, Finland, Denmark	21	*V. cholerae* (non-O1/O139), *V. vulnificus*, *V. alginolyticus*, *Vibrio damsela*	Baker-Austin et al. (2013)
1998	Finland, Denmark	6	*V. cholerae* (non-O1/O139)	Baker-Austin et al. (2013)
1999	Finland	8	*V. cholerae* (non-O1/O139), *V. fluvialis*	Baker-Austin et al. (2013)
2000	Finland	5	*V. cholerae* (non-O1/O139), *V. parahaemolyticus*	Baker-Austin et al. (2013)
2001	Finland, Sweden	11	*V. cholerae* (non-O1/O139), *V. vulnificus*, *V. parahaemolyticus*	Baker-Austin et al. (2013)
2002	Germany, Finland	10	*V. cholerae* (non-O1/O139), *V. vulnificus*	Baker-Austin et al. (2013)
2003	Finland, Germany, Sweden	19	*V. cholerae* (non-O1/O139), *V. vulnificus*	Baker-Austin et al. (2013)
2004	Finland, Sweden	6	*V. cholerae* (non-O1/O139), *V. parahaemolyticus*, *Vibrio* spp.	Baker-Austin et al. (2013)
2005	Finland, Sweden	9	*V. cholerae* (non-O1/O139), *V. parahaemolyticus*, *Vibrio* spp.	Baker-Austin et al. (2013)
2006	Germany, Denmark, Sweden, Poland Finland, Estonia, (Netherlands)	67	*V. cholerae* (non-O1/O139), *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*, *Vibrio* spp.	Baker-Austin et al. (2013)
2007	Finland, Sweden	14	*V. cholerae* (non-O1/O139), *Vibrio* spp.	Baker-Austin et al. (2013)
2008	Sweden, Finland, (Netherlands)	13	*V. vulnificus*, *Vibrio* spp.	Baker-Austin et al. (2013)
2009	Finland, Sweden, Germany, (Netherlands)	15	*V. cholerae* (non-O1/O139), *V. parahaemolyticus*, *V. alginolyticus*, *Vibrio* spp.	Baker-Austin et al. (2013)
2010	Finland, Sweden, Germany, (Netherlands)	36	*V. cholerae* (non-O1/O139), *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*	Baker-Austin et al. (2013)
2011	United Kingdom	1	*V. alginolyticus*	Reilly et al. (2011)
2014	Finland, Sweden	89	*V. cholerae* (non-O1/O139), *V.parahaemolyticus, V. vulnificus, V. alginolyticus, Vibrio spp.*	Baker-Austin et al. (2016)

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