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VIBRIO SPP. IN THE GERMAN BIGHT

by

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TABLE OF CONTENTS

INTRODUCTION 1

RESEARCH AIMS 8

OUTLINE 10

CHAPTER I 13
A polyphasic approach for the differentiation of environmental Vibrio *isolates from temperate waters*.

CHAPTER II 41
Occurrence of Vibrio parahaemolyticus *and* Vibrio alginolyticus *in the German Bight over a seasonal cycle*.

CHAPTER III 67
Seasonal dynamics and predictive modeling of a Vibrio *community in coastal waters of the North Sea*.

GENERAL DISCUSSION 93

SUMMARY 103

REFERENCES 105

ACKNOWLEDGEMENTS 128

DECLARATION 131

INTRODUCTION

Region in focus: The North Sea

The North Sea is a semi-enclosed shelf sea, which stretches between the Northern European mainland and the Atlantic Ocean. It has an average depth of 95 m with a maximum of 750 m in the south of Norway. The average water temperature is 6°C in winter and 17°C in summer. North Sea water has a salinity of 34 – 35, but the salinity around river discharges is much lower (MUMM, 2000, Howarth, 2001). The North Sea is constantly in motion in response to tides, wind and the inflow of Atlantic Ocean water or freshwater, with tides representing the strongest influence. Some parts of the northern North Sea stratify in spring and autumn due to solar heat input, whereas the southern North Sea and particularly the German Bight represent usually a well mixed system throughout the year due to its shallow depths and the strong tidal influence (Howarth, 2001).

The North Sea is exposed to many anthropogenic influences in terms of shipping lanes, harbors, fishery industry, tourism, recreation and the use of energy resources. Moreover, the climate change has a great influence in the North Sea. For instance, significant changes of temperature, light and nutrient conditions have been detected over the last 45 years in the German Bight, the sampling region of this study (Wiltshire*, et al.*, 2010). These dynamics affect the whole food web, particularly the organisms at the base of the food web.

Bacteria and viruses do not only represent the smallest members of the marine ecosystem, but also the ones who adapt fastest to their environment. Differences of bacterial communities regarding biotic and abiotic factors including seasonal dynamics have already been detected in the German Bight (Sapp*, et al.*, 2007, Boer*, et al.*, 2009) and a community shift due to climate change can be expected.

One bacterial group, which benefits in particular from the consequences of climate change, such as increasing water temperatures, is the genus *Vibrio* with its many mesophilic representatives.

The genus *Vibrio*

The first description of a *Vibrio* species took place in 1854, when the Italian physician Pacini discovered *V. cholera*. The latest version of the Bergey's Manual of Systematic Bacteriology includes 44 fully described *Vibrio* species (Farmer*, et al.*, 2005). The

genus *Vibrio* represents a very heterogeneous group of bacteria and a subdivision of the genus has been proposed (Thompson, *et al*., 2004a, Farmer, *et al*., 2005). To date *Vibrio* bacteria are characterized as gram-negative, oxidase-positive, facultative anaerobe bacteria with motile rods (Farmer, *et al.*, 2005). They are chemoorganotroph and capable of fermentative and respiratory metabolism. *Vibrio* bacteria are mainly mesophilic and primarily aquatic. They occur commonly in marine and estuarine waters and sediments and in the intestinal contents of marine organisms. Twelve species have been detected in human clinical specimens and have been reported to cause intestinal or extraintestinal infections (Farmer & Hickman-Brenner, 2006).

	Occurrence in human clinical specimens	
	Intestinal	Extraintestinal
V. alginolyticus	+	++
V. carchariae	-	+
V. cholerae		
Serogroup O1	++++	+
Serogroup non O1	++	++
V. cincinnatiensis	-	+
V. damsela	-	++
V. fluvialis	++	-
V. furnissii	++	-
V. hollisae	++	-
V. metschnikovii	-	+
V. mimicus	++	+
V. parahaemolyticus	++++	+
V. vulnificus	+	+++

TABLE 1. Infection causing *Vibrio* species and their location in human clinical specimens (according to Farmer & Hickman-Brenner, 2006);
The symbols +, ++, +++, ++++ display relative frequency of corresponding species in human specimens.

Vibrio spp. have several strategies to survive unfavorable environmental conditions, such as nutrient depletion or cold temperatures. By the formation of biofilms, *Vibrio* bacteria can attach to algae, crustaceans or other marine organisms, and utilize the nutrients released by these organisms (Huq, *et al.*, 1983, Snoussi, *et al.*, 2008, Visick, 2009, Yildiz & Visick, 2009). Another survival strategy of *Vibrio* spp. is to enter a viable but non-culturable (VBNC) state (Roszak & Colwell, 1987, Asakura, *et al.*, 2007). Bacteria in a VBNC state do not form colonies on any media, but are metabolically active and very resistant to environmental stress (Wong & Wang, 2004, Oliver, 2005). Two chromosomes are common to all *Vibrio* spp., one large and one small more flexible chromosome (Okada, *et al.*, 2005). High rates of mutation, recombination and frequent horizontal gene transfer lead to high polymorphism and intraspecies variability of *Vibrio* spp. (Moreno, *et al.*, 2002, Gonzalez-Escalona, *et al.*,

2005, Hazen, *et al.*, 2010). This high genome plasticity enables these bacteria to adapt to various niches and react rapidly to environmental changes.

Several environmental parameters have been shown to influence *Vibrio* communities. The main driving factors seem to be temperature, salinity and phytoplankton occurrence (Drake, *et al*., 2007, Hsieh, *et al*., 2008, Martinez-Urtaza, *et al*., 2008b, Turner, *et al*., 2009, Vezzulli, *et al*., 2009). Especially high water temperature has been reported to correlate with increased *Vibrio* abundance (Blackwell & Oliver, 2008, Soto, *et al*., 2009, Caburlotto, *et al*., 2010b). Therefore, *Vibrio* bacteria are likely to gain in importance in the course of climate change, also in European waters.

So far, some species such as *V. alginolyticus* or *V. splendidus* have been detected in Northern as well as Southern Europe (Thompson, *et al*., 2004b, Hidalgo, *et al*., 2008, Schets, *et al*., 2010, Vezzulli, *et al*., 2010). Other *Vibrio* species, for instance *V. anguillarum* and *V. aestuarianus*, are rather adapted to colder water temperatures and occur mainly in the North and Baltic Sea (Eiler, *et al.*, 2006). The potentially human pathogenic species *V. parahaemolyticus*, *V. vulnificus* and *V. cholera* have been detected in both, the North (Bauer, *et al.*, 2006, Lhafi & Kühne, 2007) and South of Europe (Gugliandolo, *et al.*, 2009, Vezzulli, *et al.*, 2009, Rodriguez-Castro, *et al.*, 2010), with a higher frequency in southern waters such as the Mediterranean Sea. In general, only some strains of those potentially pathogenic species in European waters have been reported to be infectious and to carry virulence factors.

Human pathogenic *Vibrio* spp. in temperate European waters

Vibrio infections have been more common in tropical areas, but over the last decade they are also emerging in European regions (Gras-Rouzet, *et al.*, 1996, Martinez-Urtaza, *et al.*, 2005, Cooke & Shapiro, 2007).

Although pathogenic *Vibrio* strains seem to be spreading geographically and *Vibrio* caused infections are reported more frequently, no regulated monitoring for *Vibrio* spp. exists in European waters. Previous studies from temperate European waters mainly focused on the aspect of seafood safety with quantification and detection of *Vibrio* spp. in general (Hoi, *et al.*, 1998, Bauer, *et al.*, 2006, Lhafi & Kühne, 2007) or on the genomic diversity of a certain *Vibrio* species (Ellingsen, *et al.*, 2008, Rodriguez-Castro, *et al.*, 2010). So far, the occurrence and dynamics of potentially pathogenic *Vibrio* species and their pathogenicity factors in European waters are not fully understood and therefore difficult to predict. Since seawater and sediments can serve as a reservoir for

pathogenicity vectors, all existing European studies on pathogenic *Vibrio* spp. stress the risk of further spreading of pathogenic strains and the importance of further investigations.

Especially *V. vulnificus* and *V. parahaemolyticus* represent an increasing medical threat in Europe (Baker-Austin*, et al.*, 2010). *V. vulnificus* and *V. parahaemolyticus* strains carrying pathogenicity factors occur more frequently in southern than in northern European waters (Baffone, *et al*., 2006, Martinez-Urtaza, *et al*., 2008b, Caburlotto, *et al*., 2010a). But reports of infectious *V. vulnificus* and *V. parahaemolyticus* strains increase also in northern European waters, namely in the North and Baltic Sea (Fouz*, et al.*, 2006, Cooke & Shapiro, 2007). *V. vulnificus* have mainly been found in the Baltic Sea, whereas *V. parahaemolyticus* is the main potential *Vibrio* pathogen in the North Sea (Ruppert, *et al*., 2004, Schets, *et al*., 2010). *V. parahaemolyticus* is also known to occur in the German Bight, the sampling region of this thesis, but profound studies on this potential pathogen in the German Bight are lacking. Therefore, this thesis focuses mainly on the abundance and ecology of the species *V. parahaemolyticus* in the German Bight (North Sea, Germany).

The species *Vibrio parahaemolyticus*

V. parahaemolyticus is one of the twelve *Vibrio* species occurring in human clinical specimens. It was first discovered in 1950 by Fujino during a severe foodborne disease outbreak in Osaka, Japan (Fujino*, et al.*, 1974). *V. parahaemolyticus* is a halophilic bacterium with a temperature optimum of 37°C and a pH optimum of 7.8 -8.6 (Oliver & Kaper, 1997, Farmer & Hickman-Brenner, 2006). It is autochthon in marine waters and can be ingested by humans via contaminated seafood.

Pathogenic strains of *V. parahaemolyticus* primarily cause gastroenteritis with abdominal pain, vomiting and diarrhea. *V. parahaemolyticus* represents one main cause for foodborne gastroenteritis, especially in Asia and the United States (Su & Liu, 2007). According to the U.S. Food and Drug Administration the critical level for *V. parahaemolyticus* consumption is 10^4 N x g^{-1} (USFDA, 2001a, USFDA, 2005).

Only a minority of environmental *V. parahaemolyticus* strains carries pathogenicity factors and causes infections. The pathogenicity mechanism of *V. parahaemolyticus* involves several proteins, enzymes and other virulence factors and is so far not completely understood. The earliest finding concerning the pathogenicity of *V. parahaemolyticus* was the observation that hemolytic strains develop a halo on blood

agar, the so-called "Kanagawa Phenomenon" (Wagatsuma, 1968). We know by now that the Kanagawa reaction is attributed to the thermostable direct hemolysin protein (TDH) (Nishibuchi & Kaper, 1985, Nishibuchi & Kaper, 1995). Also several pathogenic Kanagawa-negative *V. parahaemolyticus* strains have been reported. The pathogenicity of those strains has been attributed to the gene *trh* (encoding the TDH-related hemolysin) (Honda*, et al.*, 1989). In recent years, the use of cutting-edge molecular methods has revealed the complexity of the pathogenicity mechanism of *V. parahaemolyticus*. Pathogenicity islands on the smaller chromosome have been identified and several genes, such as the genes encoding the type III secretion system, have been reported to be involved in the pathogenicity mechanism (Okura, *et al*., 2003, Sugiyama *et al*., 2008, Izutsu, *et al*., 2008, Caburlotto, *et al*., 2009).

Not only is the knowledge about *V. parahaemolyticus* increasing, but also the reports of spreading pathogenic strains are increasing. In the last decade, several pandemic *V. parahaemolyticus* serotypes caused gastroenteritis epidemics worldwide (Honda*, et al.*, 2008). All these pandemic strains had a specific open reading frame (ORF8) in common, which derived from the filamentous phage f237 (Nasu*, et al.*, 2000, Iida*, et al.*, 2001). Especially the pandemic clone O3:K6 gained attention, since it spread all over the globe within 10 years and caused severe gastroenteritis worldwide (Drake*, et al.*, 2007, Nair*, et al.*, 2007). It was first detected in 1996 during a gastroenteritis outbreak in Calcutta, India (Okuda*, et al.*, 1997). Since then it has spread from Asia to the United States (Daniels, *et al*., 2000), Central and South America (Gonzalez*, et al.*, 2005, Cabanillas-Beltrán*, et al.*, 2006) and Africa (Ansaruzzaman*, et al.*, 2005). The recent detection of the serotype O3:K6 in Spain, Italy and France (Martinez-Urtaza*, et al.*, 2005, Quilici*, et al.*, 2005, Ottaviani*, et al.*, 2008) emphasizes the emerging significance of pathogenic *V. parahaemolyticus* strains in European waters.

Methodological approaches for investigations on *V. parahaemolyticus*

Currently, a large number of cultivation and molecular methods are applied worldwide to quantify and characterize potentially pathogenic *Vibrio* spp., but no standardized or international acknowledged approach has been defined. In the following, some frequently applied methods are introduced briefly.

To quantify potentially pathogenic *Vibrio* spp. in seafood, "most probable number" (MPN) procedures are used (Drake*, et al.*, 2007). The material in question is homogenized and enriched in alkaline peptone water (APW) for 24 h at 37°C. Samples from tubes positive for growth are streaked onto *Vibrio* specific agar, such as thiosulfate-citrate-bile-sucrose (TCBS) agar. Based on their specific metabolism products, different *Vibrio* species grow with different colony morphologies on TCBS agar (Figure 1).

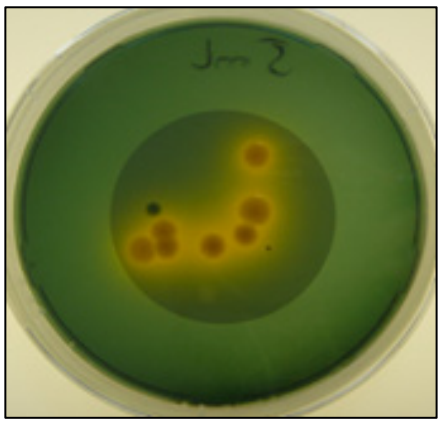

FIGURE 1. Membrane filter with concentrated water sample on TCBS agar plate; Due to their colony morphology, bacteria can be assigned to *V. alginolyticus* (yellow) and *V. parahaemolyticus* (green)

For the isolation of *V. parahaemolyticus* a recently discovered chromogenic medium (CHROMagar) was shown to be very efficient and accurate (Hara-Kudo*, et al.*, 2001, Di Pinto*, et al.*, 2011). Seawater samples are usually concentrated directly on membrane filters, which are placed on *Vibrio* specific agar as well. Colonies are counted after a 24h incubation at 37°C and colony forming units (CFU) are calculated.

The characterization of the isolates is carried out biochemically or, with the development of new methods, molecular biologically. For instance, colony hybridization (Nishibuchi*, et al.*, 1986, Yamamoto*, et al.*, 1992) or direct plate hybridization (Gooch*, et al.*, 2001) are applied to identify *V. parahaemolyticus* or certain virulence-associated genes of *V. parahaemolyticus*. Also Matrix-assisted laser desorption / ionization time-of-flight mass spectrometry (MALDI-TOF MS) has also been proposed as screening method for *V. parahaemolyticus* isolates (Hazen, *et al.*, 2009b). When it comes to the phylogenetic classification of *Vibrio* isolates, sequencing of the gene *rpoB* (encoding the RNA polymerase beta subunit) has proven to be a more reliable tool than 16S rRNA sequencing (Ki*, et al.*, 2009). To confirm the identity of *V. parahaemolyticus*, several PCR approaches have been developed, targeting species specific genes such as *tlh*, *gyrB* and *toxR* or the DNA region pR72H (Drake*, et al.*, 2007).

Only some *V. parahaemolyticus* strains carry pathogenicity factors and are pathogenic to humans. To discriminate between pathogenic and non-pathogenic strains, PCRs targeting virulence-associated markers such as *tdh*, *trh* or ORF8 are applied (Drake*, et al.*, 2007). The screening for species-specific markers is often connected to the screening for virulence–associated markers in terms of multiplex-PCRs (Bej*, et al.*, 1999, Panicker*, et al.*, 2004). Some multiplex-PCRs combine species-specific markers of several *Vibrio* pathogens such as *V. parahaemolyticus*, *V. cholera* and *V. vulnificus* (Brasher, *et al*., 1998, Bauer & Rørvik, 2007, Tarr, *et al*., 2007). In recent years, several real-time PCR protocols have been published to estimate the number of total and pathogenic *V. parahaemolyticus* in seafood sources (Takahashi*, et al.*, 2005, Nordstrom*, et al.*, 2007). But thus far, enrichment cultures are usually used for these PCR applications, since the output of the targeted *V. parahaemolyticus* genes from water or seafood samples directly is often below the detection threshold (Dileep*, et al.*, 2003, Drake*, et al.*, 2007).

More traditional pathogenicity tests include the urease test (color change of urea broth proves the presence of the gene *ure*, which is closely linked to the gene *trh*) and the hemolysis test (halo around colonies on blood agar proves hemolytic activity). Both tests are relatively time and material consuming and are about to be replaced by the above mentioned molecular-based methods.

To get further insights into the intra-species variability of *V. parahaemolyticus* strains, successful applied typing methods include ribotyping, multilocus sequence typing, repetitive sequence-based PCRs (e.g. ERIC, REP) and pulsed-field gel electrophoresis (PFGE) (Marshall*, et al.*, 1999, Maluping*, et al.*, 2005, Gonzalez-Escalona*, et al.*, 2008, Kam*, et al.*, 2008).

Although many methods exist to quantify and characterize *V. parahaemolyticus*, ongoing examination, the definition of an international standardized methodological approach and the constant development of new methods to investigate this highly adaptable bacterium are essential.

RESEARCH AIMS

The aim of this thesis was to study the *Vibrio* community at Helgoland Roads (North Sea, Germany) from a wide range of angles. *Vibrio* bacteria are not integrated into a regular monitoring and only a few studies exist with the focus on potentially pathogenic *Vibrio* species in the North Sea. Therefore, many questions regarding *Vibrio* spp. in the North Sea, their abundance, characterization, pathogenicity and response to changing environmental parameters are unresolved. The current study aimed to answer some of the main questions with special emphasis on the species *V. parahaemolyticus*, a potential pathogen emerging in the North Sea. The priorities were on the following four points:

Method evaluation

A large number of methods to characterize *Vibrio* spp. are frequently applied all over the world, but no standardized and internationally recognized approach has been defined thus far. This study aimed to evaluate a broad range of methods to reliably characterize closely related *Vibrio* species, such as *V. parahaemolyticus* and *V. alginolyticus*. We aimed to identify, which genes are the most reliable markers for the identification of the species *V. parahaemolyticus* and its pathogenicity and which fingerprinting method reveals the highest discriminatory power regarding the intraspecific diversity of *V. parahaemolyticus* and its close relative *V. alginolyticus*. The final goal was to define a hierarchical approach combining the methods proven to be most reliable, least expensive and least labor-intensive.

Quantification

Only little information is available about the abundance of *Vibrio* spp. in the water column and in shellfish of the North Sea. To investigate on this highly relevant topic, an important aim of this study was to quantify free-living, plankton-attached and shellfish-associated *Vibrio* spp. at Helgoland Roads. We intended to record the numbers of the whole *Vibrio* community at Helgoland Roads using a cultivation-independent approach, whereas we aimed to quantify selectively the potentially pathogenic *Vibrio* spp. using a cultivation approach.

Characterization

Beyond the quantification of the *Vibrio* community, it was a major point of interest within this thesis to identify the dominating *Vibrio* species at Helgoland Roads and possibly present pathogenicity factors. During a two-year sampling period, *Vibrio* spp. isolates were cultivated and a culture collection was created. Using the most reliable methods, we aimed to classify these *Vibrio* isolates phylogenetically, detect their pathogenicity and get an insight into the intraspecific diversity of *Vibrio* spp. at Helgoland Roads. Special emphasis was put on the potentially pathogenic species *V. parahaemolyticus* and its close relative *V. alginolyticus*.

Estimation of effects of environmental parameters

Recently, a spread of pathogenic *V. parahaemolyticus* strains to temperate European waters has been observed. A possible factor involved in this spread is the overall rising water temperature in the course of climate change. Most likely, other parameters play a role as well. But so far, little is known about the effects of environmental parameters on *Vibrio* communities in temperate European waters. In this study we aimed to elucidate how biotic and abiotic factors in the North Sea affect *Vibrio* spp. in general and the species *V. parahaemolyticus* in particular.

OUTLINE

The present thesis consists of a general introduction, three chapters representing one manuscript each and a general discussion.

Manuscript I (published in *FEMS Microbial Ecology*)
Oberbeckmann S, Wichels A, Maier T, Kostrzewa M, Raffelberg S & Gerdts G (2011) **A polyphasic approach for the differentiation of environmental *Vibrio* isolates from temperate waters.** *FEMS Microbiol Ecol* **75** (1): 145-162.
This manuscript represents the evaluation of several frequently applied classification methods regarding the usability to identify *Vibrio* isolates. The outcome is the definition of a reliable approach to differentiate even between closely related *Vibrio* species. The laboratory investigations were carried out by Sonja Oberbeckmann and the diploma student Sarah Raffelberg. The MALDI-TOF MS analyses were performed by Thomas Maier and Markus Kostrzewa (Bruker Daltonics). The planning, evaluation and manuscript writing was carried out by Sonja Oberbeckmann under the guidance of Antje Wichels and Gunnar Gerdts.

Manuscript II (submitted to *Antonie van Leeuwenhoek Journal of Microbiology*)
Oberbeckmann S, Wichels A, Wiltshire KH & Gerdts G **Occurrence of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* in the German Bight over a seasonal cycle.**
This manuscript describes the quantification and classification of mesophilic *Vibrio* spp. from seawater, plankton and shellfish samples using a cultivation approach. The influence of environmental parameters on certain *Vibrio* species is also presented. The planning, the laboratory investigations and the manuscript writing were accomplished by Sonja Oberbeckmann with the assistance of Antje Wichels and Gunnar Gerdts. The environmental data were provided by Karen H. Wiltshire.

Manuscript III (submitted to *Microbial Ecology*)

Oberbeckmann S, Fuchs B, Meiners M, Wichels A, Wiltshire KH & Gerdts G **Seasonal dynamics and predictive modeling of a *Vibrio* community in coastal waters of the North Sea.**

In this manuscript the implementation and results of a cultivation-independent approach to investigate a *Vibrio* community are presented. *Vibrio* spp. numbers and their responses to environmental parameters are reported and multiple regression models are described. The laboratory investigations were performed by Mirja Meiners under the guidance of Bernhard Fuchs and the environmental data were provided by Karen H. Wiltshire. The statistical calculation, evaluation and manuscript writing was carried out by Sonja Oberbeckmann under the guidance of Antje Wichels and Gunnar Gerdts.

CHAPTER I

A polyphasic approach for the differentiation of environmental *Vibrio* isolates from temperate waters

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Abstract

Climate change and marine traffic lead to changing species communities in the oceans. Due to increasing seawater temperatures, pathogenic *Vibrio* species could become significant even in temperate waters. We classified mesophilic *Vibrio* isolates from the German Bight (North Sea) by using a polyphasic approach with special emphasis on *V. parahaemolyticus*. MALDI TOF MS was used as a primary screen to classify isolates, 16S rRNA and *rpoB* gene sequencing to identify species. Potential *V. parahaemolyticus* isolates were screened for regulatory or virulence related genes (*toxR*, *tlh*, *tdh*, *trh*). To investigate genomic diversity, we applied repetitive-sequence based PCRs. Results were evaluated and methods compared using multivariate statistical analysis. Most isolates were classified as *V. parahaemolyticus* or *V. alginolyticus*. Reliable differentiation between both species was achieved by *rpoB* sequencing and *toxR* detection. Among the fingerprinting methods, ERIC-PCR showed the highest discriminatory power, displaying three separated clusters. These clusters represent the species *V. parahaemolyticus*, *V. alginolyticus* and one group in between. The frequent detection of *V. parahaemolyticus* in the German Bight reveals the urgency for further monitoring. In this context, a polyphasic approach, such as defined in this study, is needed to differentiate populations of *V. parahaemolyticus* and *V. alginolyticus*.

Introduction

Bacteria of the genus *Vibrio* are Gram-negative, primarily facultative anaerobes, forming motile rods (Farmer, *et al.*, 2005). They occur mainly in estuaries, marine coastal waters and sediments, free living or in association with zoo- and phytoplankton. Several *Vibrio* species are serious human pathogens causing gastroenteritis, septicemia and wound or tissue infection (Oliver & Kaper, 1997, Thompson, *et al*., 2004a, Drake, *et al*., 2007). Contact with contaminated water and consumption of raw seafood are the main infection factors for *Vibrio* associated diseases. *V. parahaemolyticus*, for instance, is one of the main causes of foodborne gastroenteritis worldwide (Yeung & Boor, 2004). Traditionally, most gastroenteritis outbreaks caused by *V. parahaemolyticus* are linked to warmer regions (Jegathesan & Paramasivam, 1976, Lesmana, *et al.*, 2001, Chowdhury, *et al.*, 2004, Sen, *et al.*, 2007). However, an increase of pathogenic *V. parahaemolyticus* can be expected in temperate waters as well: global warming leads to rising water temperatures and therefore expanded niches for these mainly mesophilic *Vibrio* species (Colwell, 1996, Paz, *et al.*, 2007). Another factor that expedites the spread of pathogenic *Vibrio* species worldwide is globalization, and associated mechanisms of mobility. For instance, due to the exchange of ballast water and sediment transported by ships, pathogenic *Vibrio* strains are distributed across the globe (Takahashi CK, McCarthy & Khambaty, 1994, Mimura, *et al.*, 2005). One highly pathogenic *V. parahaemolyticus* strain, pandemic clone O3:K6 (Nasu, *et al.*, 2000, Makino, *et al.*, 2003), caused outbreaks of severe gastroenteritis worldwide (Nair, *et al.*, 2007). It was first detected in Asia in 1996 (Okuda, *et al.*, 1997) and has since spread to the United States (Daniels, *et al*., 2000), Central and South America (Gonzalez, *et al.*, 2005, Cabanillas-Beltrán, *et al.*, 2006), Africa (Ansaruzzaman, *et al.*, 2005) and Southern Europe (Martinez-Urtaza, *et al.*, 2005, Ottaviani, *et al.*, 2008). The detection of the pandemic *V. parahaemolyticus* strain O3:K6 in Spain in 2004 and Italy in 2007 emphasizes the urgent need for suitable European monitoring. To date, studies about potential human pathogenic *Vibrio* species, such as *V. parahaemolyticus* in Northern Europe, are still rare (Eiler, *et al.*, 2006, Lhafi & Kühne, 2007, Ellingsen, *et al.*, 2008). Furthermore, a standardized detection approach for *V. parahaemolyticus* is still lacking (Croci, et al., 2007a).

MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry) is a spectrometric method to accurately determine molecular masses of,

e.g. biomolecules, such as proteins and peptides. MALDI-TOF MS has been described as a fast alternative to sequence analysis and biochemical tests for the identification and classification of bacterial isolates based on their protein composition (Fenselau & Demirev, Mellmann*, et al.*, 2008, Sauer*, et al.*, 2008). This study represents one of the first attempts to apply MALDI-TOF MS to classify *Vibrio* isolates. Prior to this study, only Hazen *et al.* (2009b) and Dieckmann *et al.* (2010) investigated *Vibrio* spp. using MALDI-TOF MS.

Sequencing of 16S rRNA and *rpoB* (gene encoding the RNA polymerase β-subunit) was applied in this study to obtain a reliable species identification of the *Vibrio* isolates. Mollet *et al.* (1997) were the first to describe *rpoB* sequence analysis as a powerful tool for bacterial species identification, which was successfully applied to *Vibrio* isolates ten years later (Tarr*, et al.*, 2007). Though databases with 16S rRNA sequences comprehensively represent the Bacteria and Archaea domains as a whole (e.g. NCBI GenBank), is the usefulness of this target as reliable species identification tool limited by the closeness between 16S rRNA sequences of many *Vibrio* species such as *V. parahaemolyticus* and *V. alginolyticus*. *RpoB* sequence analysis is likely to serve as a more resolved and reliable method of species identification (Ki, *et al.*, 2009). To complement efforts of species identification, screening of specific regulatory or virulence related genes (*toxR*, *tlh*, *tdh*, *trh*) was performed (Bauer & Rørvik, 2007). Specific PCRs screening for *tlh* and *toxR* are frequently applied to detect *V. parahaemolyticus*, and several protocols have been developed. In this context, a central issue is the differentiation of *V. parahaemolyticus* and *V. alginolyticus*, which are closely related and thus difficult to distinguish (Kita-Tsukamoto*, et al.*, 1993, Robert-Pillot*, et al.*, 2002, Xie*, et al.*, 2005).

To investigate the genomic diversity of isolated *Vibrio* strains from Helgoland Roads, we applied the repetitive-sequence based PCR methods, ERIC, BOX and REP, wherein the regions between the non-coding repetitive sequences in the bacterial genome are amplified (Stern, *et al.*, 1984, Hulton, *et al*., 1991, Versalovic, *et al*., 1991, Martin, *et al*., 1992, Wong & Lin, 2001, Maluping, *et al*., 2005). The resulting amplicon patterns are specific for each bacterial strain and can be visualized via electrophoresis as genomic fingerprints.

In our study, we hierarchically applied and assessed a wide range of methods to design a suitable approach to (1) identify and differentiate *Vibrio* species (16S rRNA / *rpoB* sequencing, MALDI-TOF MS), (2) target *V. parahaemolyticus* strains and assess their

pathogenicity (screening for *tlh*, *toxR*, *tdh* and *trh*, Kanagawa test) and (3) perform genomic fingerprinting to investigate diversity (BOX-, ERIC-, REP-PCR). Such a holistic characterization of *Vibrio* isolates could be an essential step towards more information on the nature and distribution of potentially pathogenic *Vibrio* strains in coastal European waters like the German Bight (North Sea).

Materials and methods

Sample collection

Unfiltered seawater was taken weekly with the RV Aade at Helgoland Roads (North Sea, Germany, 54°11.3′N, 7°54.0′E) from May 2008 to June 2009. Additionally, net haul samples (> 20 and > 150 µm) were taken monthly from May to October 2008 at the same sampling point. Specimen of *Mytilus edulis* shellfish were collected monthly in the South Harbor of the Island of Helgoland (54°10.5′N; 7°53.67′E) from May to October 2008. All samples were analyzed directly in the marine station Helgoland.

Sample preparation and bacteria cultivation

For the cultivation and classification of *Vibrio* isolates from different water and plankton fractions, unfiltered seawater and net haul (> 20 and > 150 µm) samples were investigated on a monthly basis. The seawater sample was filtered (10 µm) and the filtrate was concentrated on a 0.2 µm membrane filter. This fraction represents the free living and particle (< 10 µm) attached bacteria. The 20 µm net haul sample was concentrated on 20 µm gauze to investigate the phytoplankton attached bacteria. The sample from the 150 µm net haul was concentrated on 100 µm gauze to investigate the zooplankton attached bacteria. All of the above described concentrates on the 0.2 µm filter and the 20 and 100 µm gauzes were enriched in Alkaline Peptone Water (APW) (Jark & Kirschke, 2009). To selectively cultivate mesophilic and especially potentially human pathogenic *Vibrio* spp., the incubation temperature was either 37° C or 41° C. These temperatures do not cover the whole mesophilic range, nevertheless we will refer to the target bacteria of this study for simplicity as mesophilic ones. After 24 h incubation, the enrichment samples were plated in serial dilutions on TCBS (Kobayashi, *et al.*, 1963) and modified cellobiose polymyxinB colistin (mCPC) (Massad & Oliver, 1987) agar. Additionally, the complete tissue of five individual *Mytilus edulis* specimen

was collected monthly and homogenized using an Ultra-Turrax®. The homogenate was enriched in APW and the cultivation of *Vibrio* spp. was carried out on TCBS and mCPC agar as described above for plankton/seawater samples. All plates were incubated for 24 h at 37° or 41° C. As much single colonies as possible with yellow and green color (TCBS) respectively yellow and purple/blue color (mCPC) were transferred to marine broth agar containing 50 % seawater using a dilution streak. The number of transferred colonies varied over the sampling season. All resulting isolates were transferred at least three times to obtain pure cultures.

DNA extraction

The genomic DNA of the resulting isolates from seawater and shellfish was extracted using lysozyme/SDS lysis and phenol/chloroform extraction, followed by isopropanol precipitation using a modified protocol of Anderson & McKay (1983) omitting the DNA-denaturation step.

16S rRNA and *rpoB* sequencing

An approximately 1300 bp fragment of the 16S rRNA was amplified with the primer pair 63f and 1387r, as described previously (Marchesi*, et al.*, 1998). Additionally, the primer 341f (Muyzer*, et al.*, 1993) was used to obtain full sequences. A selection of 38 isolates (assigned to *V. parahaemolyticus* and *V. alginolyticus* using 16S rRNA gene sequencing) was classified using *rpoB* sequencing. An approximately 1600 bp *rpoB* fragment was amplified with the primers rpoB458F, rpoB2105R and rpoB1110F, according to Tarr *et al.* (2007) and Hazen *et al.* (2009a). Sequencing of both 16S rDNA and *rpoB* was performed using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA). The resulting sequences were aligned using AlignIR1.2® (LI-COR). In order to determine the closest relatives of the environmental isolates, the 16S rRNA gene sequences were compared with sequences in the GenBank database using the BLAST algorithm (Altschul*, et al.*, 1990).

Phylogenetic analysis

The sequences were phylogenetically analyzed using the ARB® software package (Ludwig*, et al.*, 2004). After the addition of the 16S gene sequences to the ARB 16S rRNA gene sequence database (release May 2005), the sequence alignment was carried out with the integrated Fast Aligner. Additionally, the alignment was refined by

comparison of the closest relatives in NCBI retrieved by BLAST. Sequences with more than 1200 nucleotides were used to calculate the phylogenetic tree. Partial sequences were added using the ARB 'parsimony interactive' tool. The *rpoB* gene sequences were added to an ARB *rpoB* database containing all *rpoB* sequences available in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/). After the addition, the sequence alignment was also carried out with the integrated Fast Aligner. The 1484-1593 bp long *rpoB* gene sequences were used to calculate the phylogenetic tree. The phylogenetic relationships of both, 16S rRNA and *rpoB* gene sequences, were deduced by the neighbor-joining method with the correction algorithm of Felsenstein (Felsenstein, 1993). Matrices were calculated via neighbor-joining based on the phylogenetic distances of the isolates using the 'similarity' correction.

Nucleotide sequence accession numbers

The sequences obtained in this study are available from GenBank under the accession numbers FJ952636-FJ952680 (16S rRNA) and GU301141-GU301178 (*rpoB*).

Mass spectrometry

In search of a high throughput, fast and cost-effective method to classify *Vibrio* isolates, we applied MALDI-TOF MS fingerprint analysis, as described previously for phytopathogenic bacteria (Sauer*, et al.*, 2008). We aimed to typecast the *Vibrio* isolates from Helgoland Roads by comparing their MALDI-TOF mass spectra with spectra in a database (Biotyper 2.0, Bruker Daltonics). To this purpose, all available environmental *Vibrio* spp. isolates and reference strains from an in-house culture collection (V. *parahaemolyticus* RIMD 2210633, *V. alginolyticus* DSM 2171 and *V. parahaemolyticus* DSM 2172) were grown in marine broth liquid medium for 24 h at 37° C. For storage, 2 ml of each liquid culture were centrifuged; the resulting pellet was suspended in 300 µl distilled water and mixed with 900 µl pure ethanol. The cells were extracted using a formic acid/acetonitrile method, as described previously (Mellmann*, et al.*, 2008). 1 µl of each extract was spotted onto a sample target in 20 replicates, overlaid with a HCCA (α-Cyano-4-hydroxycinnamic acid) matrix and subsequently measured in a microflex^{TM} benchtop instrument (Bruker Daltonics) in the positive linear mode (2000 to 20000 Dalton). The resulting profile spectra represent the molecular masses of a fraction of highly-abundant bacterial proteins, in particular ribosomal proteins. Bacterial isolates were classified with the MALDI Biotyper 2.0 software

(Bruker Daltonics) by comparing the obtained profiles with a database (V2.0.4.0) containing reference spectra of roughly 1900 microbial species. For further statistical analyses, the obtained molecular masses were subdivided into classes. Each class consisted of a 5 Da range. The peak intensities for each isolate were associated with the respective classes. Based on these mass classes, a similarity matrix was calculated to compare the results of the MALDI-TOF MS with the results of the other classification methods.

PCR analysis of regulatory or virulence related genes

Specific PCR for *tlh* (Taniguchi*, et al.*, 1985, Taniguchi*, et al.*, 1986), *toxR* (Bauer & Rørvik, 2007), *tdh* (Nishibuchi & Kaper, 1985) and *trh* (Honda*, et al.*, 1991, Honda & Iida, 1993) with DNA extracts from 38 environmental *Vibrio* isolates was performed. The DNA of the following strains from culture collections served as reference: *V. parahaemolyticus* RIMD 2210633, *V. parahaemolyticus* T4 and *V. parahaemolyticus* CM24 (all kindly provided by Carsten Matz, HZI) served as positive control, as they are all *tlh*- and *toxR*-positive. Furthermore, RIMD 2210633 is known to be *tdh*-positive and *trh*-negative, T4 is *tdh*- and *trh*-negative and CM24 is *tdh*- and *trh*-positive. As additional reference strains, *V. alginolyticus* DSM 2171 and *V. parahaemolyticus* DSM 2172 were used. All reactions were performed twice to confirm the results. In case of discordant results, a third PCR was carried out. The presence of the genes *tlh*, *tdh* and *trh* was determined by PCR for the *Vibrio* isolates. The PCR was performed using one of the primer sets tl-L/R, tdh-L/R or trh-L/R (Table 1). Each PCR mixture contained 5 µl Taq buffer [10x], 7.5 µl Taq Master PCR Enhancer [5x], 2 µl of each primer [20 µM], 1 µl dNTPs [10 mM], 1 U of Taq DNA polymerase (5Prime) and water to 50 µl. PCR reactions were carried out in a thermocycler (Eppendorf) according to the U.S. Food and Drug Administration`s Bacteriological Analytical Manual (BAM) (DePaola & Kaysner, 2004): Denaturation at 94° C for 3 min, followed by 25 cycles of denaturation at 94° C for 1 min, annealing at 60° C for 1 min, and extension at 68° C for 2 min, with a final extension at 68° C for 3 min. Based on preliminary experiments using different PCR protocols, we evaluated this protocol to be the most specific, which is in agreement with Croci *et al.* (2007a). The verification of the PCR products was performed as follows: 5 µl of the amplified DNA was separated in a 1.2 – 1.4 % (v/w) agarose gel by electrophoresis using 0.5x TBE. The gels were run at 100 V for 60 min.

The DNA was stained with EtBr and visualized and digitalized using an UV transilluminator (Bio-Rad).

To investigate the presence of the gene *toxR* in the *Vibrio* isolates, polymerase chain reaction (PCR) was performed according to Bauer & Rørvik (2007) with the primer set UtoxF / vptoxR (Table 1). While UtoxF is a universal primer for several *Vibrio* species, vptoxR is species-specific for *V. parahaemolyticus*. PCR reactions were run as 50 µl batches with the same composition as for the *tlh*, *tdh* or *trh* targeted PCR. The running conditions in the thermocycler (Eppendorf) were the following: Denaturation at 95° C for 4 min, followed by 25 cycles of denaturation at 95° C for 30 s, annealing at 55° C for 30 s and extension at 68° C for 7 min. The resulting PCR products were confirmed to be the expected size by agarose gel electrophoresis as described above.

TABLE 1. List of applied primer sets, primer sequences, fragment sizes and references.

Primer	Sequence (5´-3´)	Fragment (bp)	Reference
63f	cag gcc taa cac atg caa gtc	1300	Marchesi *et al*. (1998)
341f	cct acg gga ggc agc ag		Muyzer *et al*. (1993)
1387r	ggg cgg wgt gta caa ggc		Marchesi *et al*. (1998)
*rpoB*458F	agg cgt gtt ctt cga cag cga taa	1600	Hazen *et al*. (2009a)
*rpoB*1110F	gta gaa atc tac cgc atg atg		Tarr *et al*. (2007)
*rpoB*2105R	cgg cta cgt tac gtt cga tac cag		Hazen *et al*. (2009a)
tl-R	gct act ttc tag cat ttt ctc tgc	450	Taniguchi *et al*. (1985, 1986)
tl-L	aaa gcg gat tat gca gaa gca ctg		
tdh-R	tgg aat aga acc ttc atc ttc acc	270	Nishibuchi & Kaper (1985)
tdh-L	gta aag gtc tct gac ttt tgg ac		
trh-R	cat aac aaa cat atg ccc att tcc g	500	Honda & Iida (1993), Honda *et al*. (1991)
trh-L	ttg gct tcg ata ttt tca gta tct		
UtoxF	gas ttt gtt tgg cgy gar caa ggt t	300	Bauer & Rørvik (2007)
vptoxR	ggt tca acg att gcg tca gaa g		
BOX	cta cgg caa ggc gac gct gac g	diverse	Versalovic *et al*. (1994)
ERIC2	aag taa gtg act ggg gtg agc g	diverse	Versalovic *et al*. (1991)
ERIC1R	atg taa gct cct ggg gat tca c		
REP2I	icg ict tat cig gcc tac	diverse	Versalovic *et al*. (1991)
REP1R	iii icg icg ica tci ggc		

Genomic fingerprinting using rep-PCR (BOX, ERIC, REP)

The genomic fingerprints of 38 selected isolates (*V. parahaemolyticus* and *V. alginolyticus* according to 16S rRNA gene sequencing) and three reference strains (*V. parahaemolyticus* RIMD 2210633, *V. alginolyticus* DSM 2171,

V. parahaemolyticus DSM 2172) were performed using BOX-, ERIC- and REP-PCR, according to Rademaker and de Bruijn (1997). Each PCR mixture contained 0.2 µl bovine serum albumin [20 mg/ml], 2.5 µl dimethyl sulfoxide and 5 µl 5x Gitschier buffer (1 M $(NH_4)_2SO_4$, 1 M Tris-HCl pH 8.8, 1 M MgCl, 0.5 M EDTA pH 8.8, 1% mercaptoethanol). For BOX- and ERIC-PCR 1.25 µl, for REP-PCR 0.75 µl dNTPs [10 mM] were added. The mixtures contained either 1 µl of the primer BOX [20 µM] (Versalovic*, et al.*, 1994), 1 µl of each primer ERIC2 and ERIC1R (Versalovic*, et al.*, 1991), or 0.5 µl of each primer REP2I / REP1R (Versalovic*, et al.*, 1991) and water to 25 µl. The concentration of Taq DNA polymerase (5Prime) varied between 1 U (BOX, REP) and 2 U (ERIC) per mixture. The DNA concentration in the PCR mixture was either 100 ng (BOX, ERIC) or 300 ng (REP). PCR reactions were carried out in a thermocycler (Eppendorf): Initial denaturation at 95° C for 2 min, followed by 32 cycles of denaturation at 94° C for 3 sec and 92° C for 30 sec, annealing at either 50° C (BOX, ERIC) or 40° C (REP) for 1 min and extension at 68° C for 8 min. A final extension was performed at 68° C for 8 min. The visualization of the fingerprints was performed as follows: 20 µl of the PCR product was separated in a 2% (v/w) agarose gel by electrophoresis using 0.5x TBE. To achieve comparability, two lanes per gel were utilized for a 1 kb ladder (Invitrogen, Germany). The gels were run for 30 min at 100 V, followed by 180 min at 75 V. The DNA was stained with EtBr and visualized and digitalized using an UV transilluminator (Bio-Rad). All genomic fingerprints were carried out in at least three replicates to confirm the results. The evaluation and comparative analysis of the fingerprints was carried out with the BioNumerics 5.10 software (Applied Maths, Belgium). To normalize the gel images, a 1 kb ladder served as reference. Per isolate, one composite gel consisting of three fingerprint replicates was composed. In order to compare the band patterns of the isolates, band-matching analysis was performed. Based on their position, bands were assigned to classes and band-matching tables were created. Due to a preliminary evaluation (data not shown), 1% optimization and position tolerances were used for the band-matching analysis.

Kanagawa test

The 38 *Vibrio* strains and three reference strains were tested for the Kanagawa phenomenon (KP). The Kanagawa phenomenon is based on the hemolysis reaction of bacteria and can be observed on blood agar. The *Vibrio* isolates were grown for 24 h at 37° C on Wagatsuma blood agar (Wagatsuma, 1968, USFDA, 2001b) containing 5 %

washed human erythrocytes. Colonies with a β-haemolytic zone (halo) were designated as KP positive. For confirmation, the test was done in double replicates.

Statistical analysis

Multivariate statistics was performed using the subroutines MDS (multidimensional scaling), ANOSIM (analysis of similarities) and 2STAGE / RELATE (testing matched similarity matrices) of the Primer v6 software suite (PRIMER-E, Ltd., UK) (Clarke & Gorley, 2006). For that purpose, similarity matrices of the results of the three rep-PCRs (Jaccard index), the 16S rRNA and *rpoB* sequencing (ARB_dist, similarity) and the MALDI-TOF MS analysis (Ochiai index) were compiled. Prior to similarity calculations of the MALDI-TOF MS results, the mass intensities were square root transformed. The results of all methods were subjected to factor analysis: (i) classification according to GenBank BLAST and MALDI-TOF MS, (ii) assignment into phylogenetic groups (ARB), (iii) the presence/absence of *toxR*, *tlh* and (iv) the Kanagawa phenomenon. Ordination of the similarities of the 38 *Vibrio* isolates and three reference strains was carried out by MDS. Due to clearness, all plots are presented 2-dimensional. ANOSIM was performed to test the hypothesis that the similarity within the groups was greater than between the groups. To examine the relations between the applied classification methods, the similarity matrices of the results of the rep-PCRs, the 16S rRNA and *rpoB* gene sequencing and the MALDI-TOF MS analysis were compared by 2STAGE and RELATE using the Spearman correlation.

Results

Analysis of 16S rRNA gene sequences

We sequenced the 16S rRNA gene of 84 isolates, and 44 isolates could be identified as *Vibrio* spp.. Based on 16S rRNA gene sequencing, 29 out of these 44 (66%) *Vibrio* isolates from Helgoland Roads could be assigned to the species *V. parahaemolyticus* by GenBank BLAST (Table 2). Eight isolates were classified as *V. alginolyticus* (18%), three as *V. harveyi* (7%). Four *Vibrio* isolates (9%) could not clearly be assigned to a specific *Vibrio* species. All isolates from Helgoland Roads were 99-100% similar to known strains in the GenBank database. Several *V. parahaemolyticus* isolates showed high phylogenetic conformity to the pandemic strain RIMD 2210633 (serotype O3:K6) at the 16S rRNA level. The 16S rRNA genes of the DSM strains 2171 and 2172, according to the DSMZ designated as *V. alginolyticus* (DSM 2171) and *V. parahaemolyticus* (DSM 2172), were also sequenced. But our sequence analysis revealed no distinct assignment of these strains to *V. alginolyticus* or *V. parahaemolyticus*. A phylogenetic tree of the *Vibrio* isolates from Helgoland Roads and their next relatives was created using ARB (Figure 1). The 44 *Vibrio* isolates from Helgoland Roads were assigned to two distinct groups within the 16S rRNA ARB tree. One group consisted of *V. harveyi* strains (*V. harveyi* group). The other group illustrated the close relatedness of *V. parahaemolyticus* and *V. alginolyticus* (*V. alginolyticus* / *parahaemolyticus* group): Reference strains from both species were positioned very close together in the tree. Isolates assigned to *V. alginolyticus* using GenBank BLAST, were mainly located in the upper part of the tree and isolates, assigned to *V. parahaemolyticus,* were rather located in the bottom. However, a significant distinction between *V. alginolyticus* and *V. parahaemolyticus* isolates could not be achieved using 16S rRNA analysis.

Analysis of *rpoB* sequences

Recent studies postulate a reliable classification of *Vibrio* isolates using *rpoB* sequencing rather than 16S rRNA gene sequencing (Tarr*, et al.*, 2007, Ki*, et al.*, 2009). To get a higher phylogenetic resolution, especially between *V. alginolyticus* und *parahaemolyticus* isolates, we applied *rpoB* sequencing to the 38 isolates *V. alginolyticus* / *parahaemolyticus* group of the 16S rRNA analysis. The *rpoB* sequences were analyzed using ARB and a phylogenetic tree was created including

closely related *rpoB* sequences in GenBank (Figure 2). Based on this tree, the *Vibrio* isolates from Helgoland Roads were assigned to three groups. Of the 38 isolates, 11 strains (29%) were located in a group surrounded exclusively by *rpoB* sequences of *V. parahaemolyticus* strains ('*Vibrio* group I'). The other 27 (71%) isolates fell into two groups ('*Vibrio* groups IIa / IIb'), which were clearly distinct from group I. Besides *rpoB* sequences from Helgoland strains, *Vibrio* group IIb contained solely *rpoB* sequences from *V. alginolyticus* strains. According to its position in the tree, group IIa was closely related to group IIb.

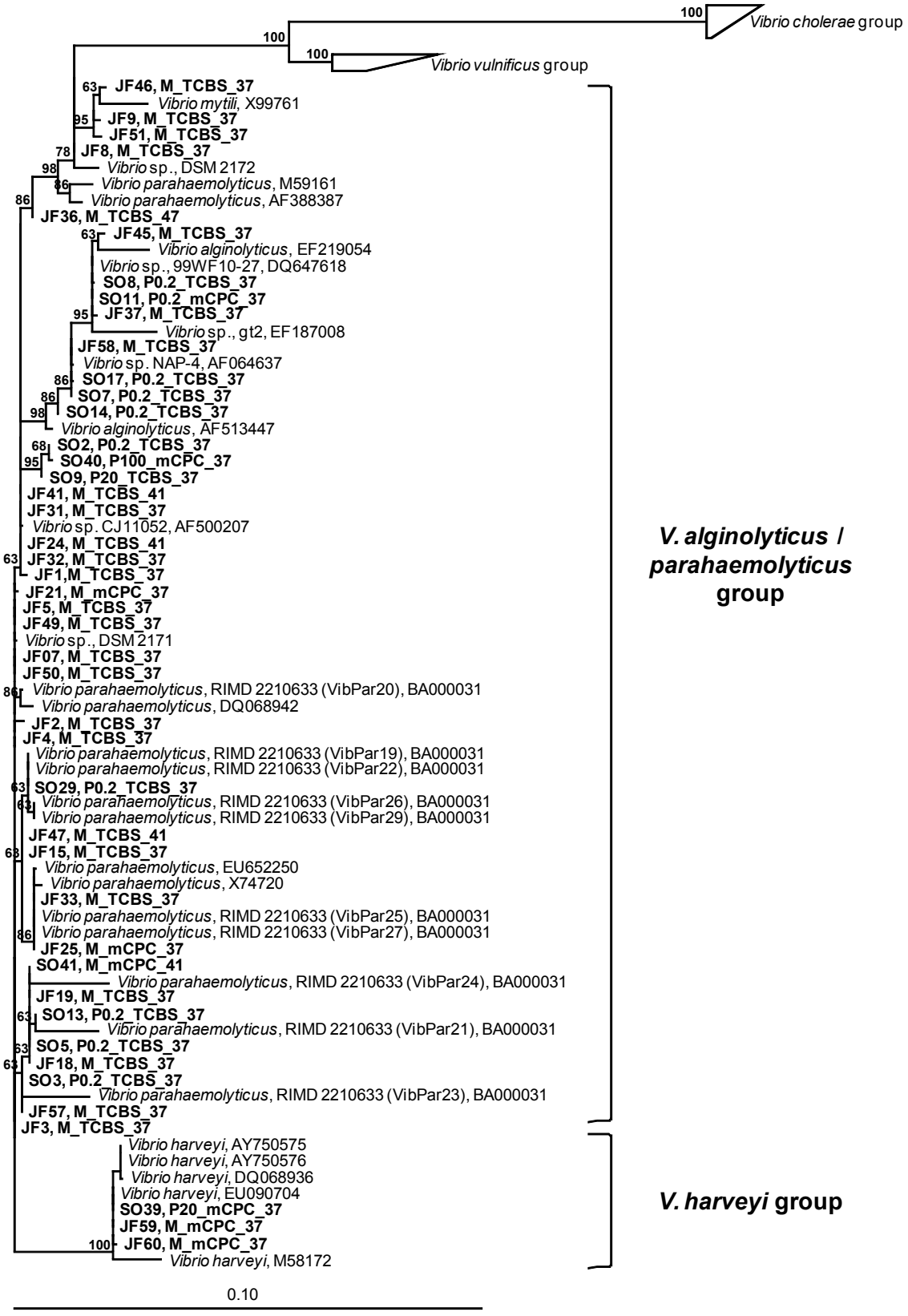

FIGURE 1. Phylogenetic tree of members of the genus *Vibrio* based on 16S rRNA sequences; indicated in bold: strains from Helgoland (SO/JF) isolated from *Mytilus edulis* (M) or different plankton fractions (P0.2/P20/P100), grown on TCBS/mCPC agar at 37°/41°C. GenBank accession numbers are given. Bootstrap values >50% are displayed. Scale bar represents 10 nucleotide substitutions per 100 nucleotides.

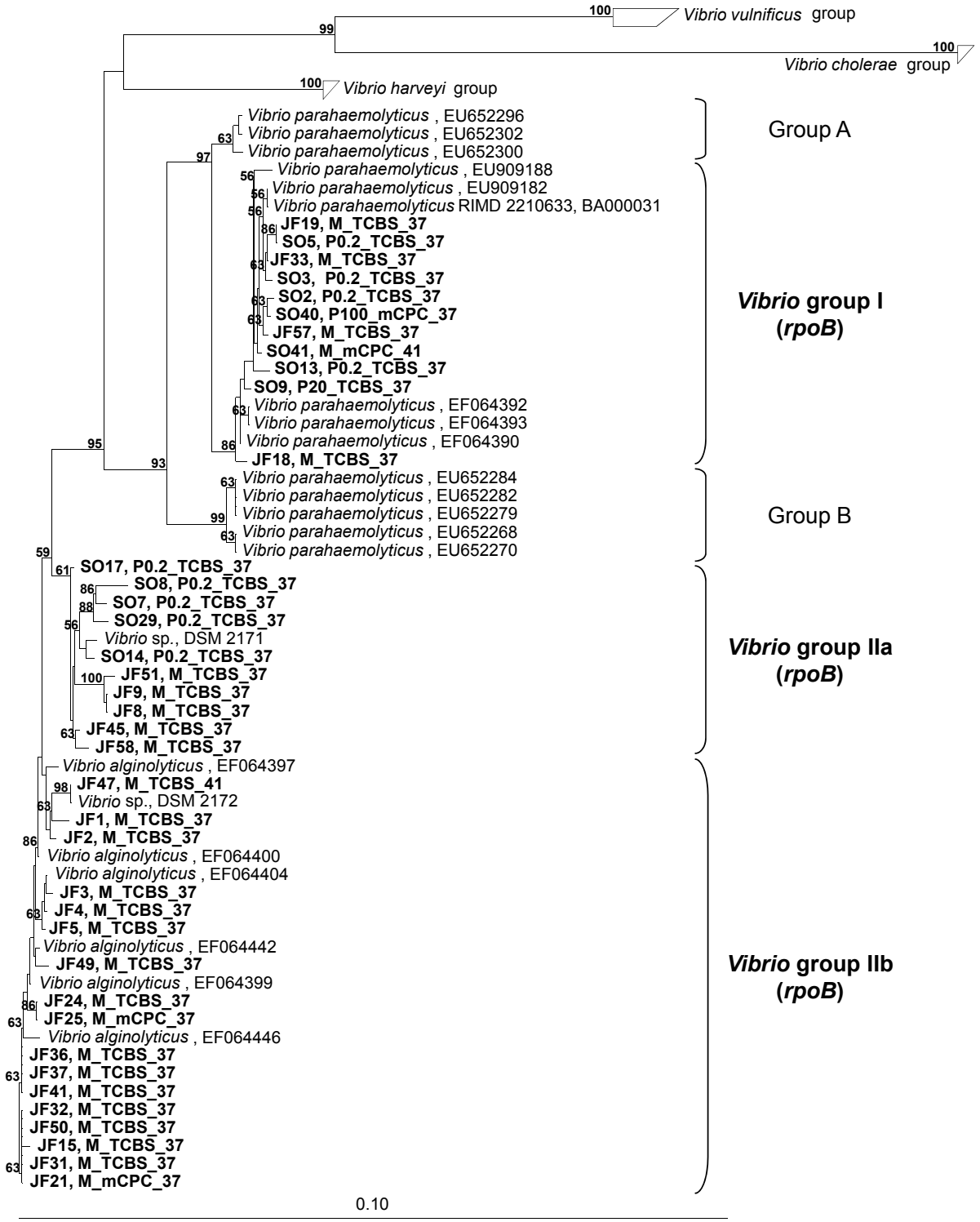

FIGURE 2. Phylogenetic tree of members of the genus *Vibrio* based on *rpoB* sequences; indicated in bold: strains from Helgoland (SO/JF) isolated from *Mytilus edulis* (M) or different plankton fractions (P0.2/P20/P100), grown on TCBS/mCPC agar at 37°/41°C. GenBank accession numbers are given. Bootstrap values >50% are displayed. Scale bar represents 10 nucleotide substitutions per 100 nucleotides. Groups A/B were omitted from further analysis (containing no strains of actual study).

Mass spectrometry

47 *Vibrio* strains (44 environmental and three reference strains) were analyzed by MALDI-TOF mass spectrometry fingerprinting. With the exception of a single *V. harveyi* isolate, all environmental isolates could be classified with confidence scores greater than 2.0 (cut-off for confident genus and probable species identification according to Bruker Daltonik). 37 environmental isolates were analyzed with a score higher than 2.3, which expresses highly probable species identification. From the 44 environmental isolates, 30 were classified as *V. alginolyticus* (68%) and 12 as *V. parahaemolyticus* (27%). Two isolates were identified as *V. trachuri* (5%). The MALDI-TOF classification results are displayed in Table 2.

Detection of specific regulatory or virulence related genes

For the specific identification of the potential pathogen *V. parahaemolyticus*, we PCR-targeted the regulatory *tlh* and *toxR* genes for 38 isolates, which were assigned to *V. alginolyticus* and *V. parahaemolyticus* (based on 16S rRNA and *rpoB* analyses). To evaluate the potential pathogenicity of *Vibrio* isolates, we additionally performed specific PCR for the virulence related genes *tdh* and *trh*. DNA from *V. parahaemolyticus* RIMD 2210633, T4 and CM24 strains served as control. The results are presented in Table 3. *Tlh* was found in 14 environmental isolates. Furthermore, a weak positive *tlh* signal could be detected in 14 other isolates. 10 *Vibrio* isolates were negative for *tlh*. Eleven isolates were positive for *V. parahaemolyticus* specific *toxR*. All 11 isolates were also positive for *tlh* and further classified as *V. parahaemolyticus* based on 16S rRNA and *rpoB* sequencing. The three *tlh* positive and *toxR* negative isolates were not classified as *V. parahaemolyticus* according to both sequencing methods. For eight environmental *Vibrio* isolates, only a weak positive *toxR* signal could be detected. *ToxR* was not detected in 19 of the isolates. Neither *tdh* nor *trh* could be detected in any of the environmental *Vibrio* isolates from Helgoland Roads.

Kanagawa test

Of all tested isolates, seven *V. parahaemolyticus* showed clear β-haemolytic activity and can therefore be referred to as Kanagawa positive. All seven Kanagawa positive isolates were positive for *tlh* as well as for *toxR*. For five other *Vibrio* isolates, only a slight β-haemolytic zone could be detected. The remaining 26 *Vibrio* isolates appeared to be Kanagawa negative.

TABLE 2. Classification of 44 environmental *Vibrio* isolates and three reference strains, based on 16S rRNA / *rpoB* sequence and MALDI-TOF analyses.

	16S rDNA analysis			*rpoB* analysis		Maldi-TOF analysis	
Strains Helgoland	**ARB Group**	**bp**	**Next relative, Coverage (BLAST)**	**ARB Group**	**Classification (ARB)**	**Next relative, Score**	**Match**
SO2	I	1270	*V. p.* J-C2-29 (EU652250), 99%	I	*V.p.*	*V. p.* DSM 10027, 2.48	+
SO3	I	1274	*V. p.* (DQ068942), 99%	I	*V.p.*	*V. p.* 4a ISB, 2.38	+
SO5	I	1234	*V. p.* J-C2-29 (EU652250), 99%	I	*V.p.*	*V. p.* DSM 10027, 2.445	+
SO9	I	1233	*V. p.* J-C2-29 (EU652250), 99%	I	*V.p.*	*V. p.* DSM 10027, 2.571	+
SO13	I	1246	*V. p.* (DQ068942), 100%	I	*V.p.*	*V. p.* DSM 10027, 2.565	+
SO40	I	1295	*V. p.* RIMD 2210633 (BA000031), 99%	I	*V.p.*	*V. p.* 4a ISB, 2.504	+
SO41	I	1304	*V. p.* RIMD 2210633 (BA000031), 99%	I	*V.p.*	*V. p.* DSM 10027, 2.539	+
JF18	I	1256	*V. p.* RIMD 2210633 (BA000031), 100%	I	*V.p.*	*V. a.* DSM 2171, 2.376	-
JF19	I	1219	*V. p.* RIMD 2210633 (BA000031), 100%	I	*V.p.*	*V. a.* DSM 2171, 2.408	-
JF33	I	1244	*V. p.* RIMD 2210633 (BA000031), 100%	I	*V.p.*	*V. a.* DSM 2171, 2.464	-
JF57	I	1280	*V. p.* RIMD 2210633 (BA000031), 100%	I	*V.p.*	*V. p.* DSM 10027, 2.362	+
SO7	I	1068	*V. sp.* 99WF10-27 (DQ647618), 99%	IIa	*V.a.*	*V. a.* DSM 2171, 2.316	+
SO8	I	1278	*V. sp.* NAP-4 (AF064637), 99%	IIa	*V.a.*	*V. a.* DSM 2171, 2.331	+
SO14	I	1284	*V. a.* RH2 (DQ664544), 99%	IIa	*V.a.*	*V. a.* DSM 2171, 2.46	+
SO17	I	1269	*V. p.* R22 (EF203212), 99%	IIa	*V.a.*	*V. a.* CCM 7037, 2.068	-
SO29	I	1278	*V. p.* (DQ068942), 100%	IIa	*V.a.*	*V. p.* DSM 10027, 2.314	-
JF8	I	866	*V. a.* (AM921804), 100%	IIa	*V.a.*	*V. a.* DSM 2171, 2.422	+
JF9	I	1264	*V. a.* (AF513447), 99%	IIa	*V.a.*	*V. p.* DSM 10027, 2.478	-
JF45	I	1288	*V. a.* H050815-1 (EF219054), 99%	IIa	*V.a.*	*V. a.* DSM 2171, 2.287	+
JF51	I	1153	*V. a.* (AF513447), 99%	IIa	*V.a.*	*V. a.* DSM 2171, 2.212	+
JF58	I	1258	*V. a.* H050815-1 (EF219054), 99%	IIa	*V.a.*	*V. a.* CCM 7037, 2.306	+
JF1	I	1300	*V. p.* (DQ068942), 99%	IIb	*V.a.*	*V. a.* DSM 2171, 2.31	-
JF2	I	1294	*V. a.* (AF513447), 99%	IIb	*V.a.*	*V. a.* DSM 2171, 2.445	+
JF3	I	1228	*V. p.* RIMD 2210633 (BA000031), 99%	IIb	*V.a.*	*V. a.* DSM 2171, 2.397	-
JF4	I	1305	*V. p.* RIMD 2210633 (BA000031), 99%	IIb	*V.a.*	*V. a.* DSM 2171, 2.408	-
JF5	I	974	*V. p.* RIMD 2210633 (BA000031), 99%	IIb	*V.a.*	*V. a.* DSM 2171, 2.252	-
JF15	I	1263	*V. p.* RIMD 2210633 (BA000031), 99%	IIb	*V.a.*	*V. p.* DSM 10027, 2.339	-
JF21	I	969	*V. p.* RIMD 2210633 (BA000031), 100%	IIb	*V.a.*	*V. a.* DSM 2171, 2.353	-
JF24	I	1287	*V. p.* (DQ068942), 99%	IIb	*V.a.*	*V. a.* DSM 2171, 2.458	-
JF25	I	917	*V. p.* RIMD 2210633 (BA000031), 100%	IIb	*V.a.*	*V. a.* DSM 2171, 2.451	-
JF31	I	1269	*V. p.* (DQ068942), 99%	IIb	*V.a.*	*V. a.* DSM 2171, 2.454	-
JF32	I	1269	*V. p.* (DQ068942), 99%	IIb	*V.a.*	*V. a.* DSM 2171, 2.497	-
JF36	I	681	*V.* sp. CJ11052 (AF500207), 99%	IIb	*V.a.*	*V. a.* DSM 2171, 2.481	+
JF37	I	710	*V. a.* (AM921804), 100%	IIb	*V.a.*	*V. a.* DSM 2171, 2.446	+
JF41	I	1264	*V. p.* (DQ068942), 99%	IIb	*V.a.*	*V. p.* DSM 10027, 2.594	-
JF47	I	1254	*V. p.* CM12 (EU660326), 100%	IIb	*V.a.*	*V. a.* DSM 2171, 2.379	-
JF49	I	681	*V. p.* J-C1-39 (EU652248), 99%	IIb	*V.a.*	*V. a.* DSM 2171, 2.311	-
JF50	I	1254	*V. p.* (DQ068942), 99%	IIb	*V.a.*	*V. a.* DSM 2171, 2.552	-
SO11	I	1272	*V. sp.* NAP-4 (AF064637), 99%			*V. a.* DSM 2171, 2.398	+
JF7	I	1004	*V. p.* RIMD 2210633 (BA000031), 99%			*V. a.* DSM 2171, 2.355	-
JF46	I	694	*V. p.* RIMD 2210633 (BA000031), 100%			*V. a.* DSM 2171, 2.446	-
SO39	II	1308	*V. h.* H050704-1 (EU090704), 99%			*V. a.* DSM 2171, 2.117	-
JF59	II	1287	*V. h.* H050704-1 (EU090704), 100%			*V. t.* LMG 19643, 1.989	-
JF60	II	1287	*V. h.* H050704-1 (EU090704), 99%			*V. t.* LMG 19644, 2.024	-
Strains CC							
RIMD2210633	I		*V. p.* (BA000031)	I	*V.p.*	2.272	+
DSMZ2172	I	1309	*V.* sp. HS2 (EU277745), 99%	IIb	*V.a.*	*V. a.* CCM 7037, 2.256	+
DSMZ2171	I	1322	*V.* sp. S3854 (FJ457562), 99%	IIa	*V.a.*	*V. a.* DSM 2171, 2.216	+

bp: base pair; *V.*: *Vibrio*, *a.*: *alginolyticus*, *p.*: *parahaemolyticus*, *h.*: *harveyi*, *t.*: *trachuri,* sp.: undefined species; CC: culture collection; score 2.30-3.00: highly probable species identification, 2.00-2.29: secure genus / probable species identification, 1.70-1.99: probable genus identification; +: consensus, -: contradiction

Strains	Group	functional genes		
Helgoland	(ARB *rpoB*)	*tlh*	*toxR*	Kanagawa
SO2	I	+	+	+
SO3	I	+	+	+
SO5	I	+	+	+
SO9	I	+	+	+
SO13	I	+	+	(+)
SO40	I	+	+	+
SO41	I	+	+	+
JF18	I	+	+	+
JF19	I	+	+	(+)
JF33	I	+	+	(+)
JF57	I	+	+	-
SO7	IIa	(+)	-	-
SO8	IIa	(+)	-	(+)
SO14	IIa	(+)	-	-
SO17	IIa	-	-	-
SO29	IIa	(+)	(+)	-
JF08	IIa	(+)	(+)	-
JF09	IIa	+	(+)	-
JF45	IIa	-	-	-
JF51	IIa	-	-	-
JF58	IIa	+	(+)	-
JF01	IIb	(+)	-	-
JF02	IIb	(+)	-	-
JF03	IIb	(+)	-	-
JF04	IIb	(+)	-	-
JF05	IIb	(+)	-	-
JF15	IIb	-	-	-
JF21	IIb	+	(+)	-
JF24	IIb	(+)	(+)	-
JF25	IIb	(+)	(+)	-
JF31	IIb	(+)	(+)	-
JF32	IIb	(+)	-	-
JF36	IIb	-	-	-
JF37	IIb	-	-	-
JF41	IIb	-	-	-
JF47	IIb	-	-	(+)
JF49	IIb	-	-	-
JF50	IIb	-	-	-
Strains CC				
RIMD 2210633	I	+	+	+
DSMZ 2172	IIb	-	-	-
DSMZ 2171	IIa	-	-	-

TABLE 3. Comparison of the results of PCRs targeting the functional genes *tlh* and *toxR* and the results of Kanagawa test for 38 environmental *Vibrio* isolates and three reference strains;
Grouping due to ARB classification (*rpoB* gene).
+: strong signal, -: no signal, (+): weak signal; CC: culture collection

Genomic fingerprinting (BOX, ERIC, REP)

To investigate the intraspecific diversity of the *V. alginolyticus* / *V. parahaemolyticus* community at Helgoland Roads, genomic fingerprinting was applied. The BOX-, ERIC- and REP-fingerprints of 38 environmental isolates from the *V. alginolyticus* / *parahaemolyticus* group of the 16S rRNA analysis were created and the resulting band patterns were analyzed. The BOX-PCR of all isolates resulted in 60 different band classes, the ERIC- and REP-PCR in 69. Based on the fingerprint pattern, the isolates could be divided in three groups, illustrated in the MDS (Figure 3).

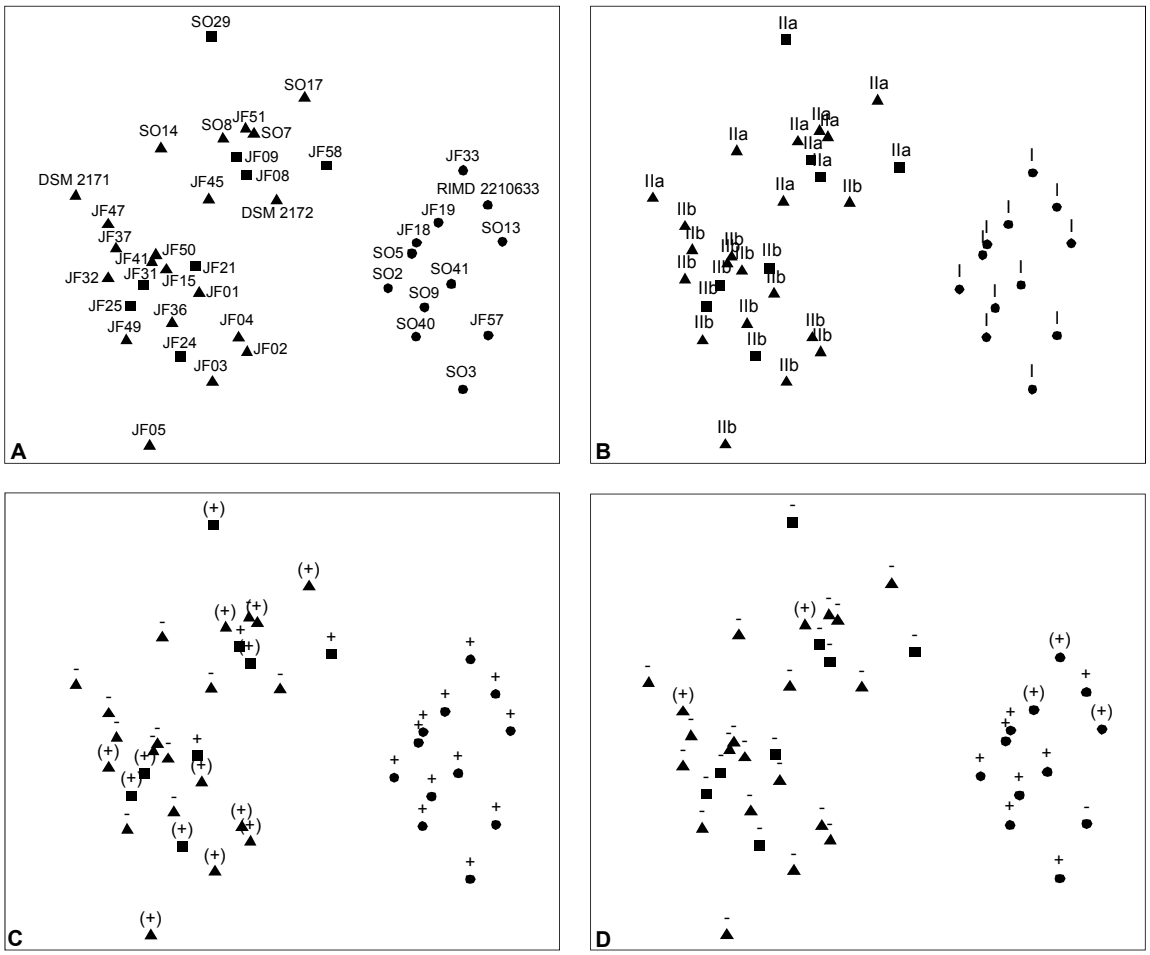

FIGURE 3. MDS plots for Jaccard similarities of ERIC fingerprints including information about the presence of *toxR*. Labels according to A: strain name (38 *Vibrio* isolates, three reference strains), B: *rpoB* grouping (I, IIa, IIb), C: presence of *tlh* and D: Kanagawa phenomenon. 2D stress value is 0.2.
+ : positive, (+) : slightly positive, -: negative; ● : *toxR* + , ■ : *toxR* (+), ▲ : *toxR* -

Method comparison using multivariate statistical analysis

Multivariate statistics was performed using the PRIMER software to compare the results of all methods (BOX-, ERIC and REP-PCR, 16S rRNA / *rpoB* sequencing, MALDI-TOF MS analysis, screening for *toxR* and *tlh* and the examination of the Kanagawa phenomenon). Based on the results of the different classification methods, the *Vibrio* isolates formed similarity groups. To test whether the similarity within these groups was greater than between the groups, ANOSIM analysis was performed using global and pairwise tests (Tables 4 and 5). Of the classification methods (16S rRNA and *rpoB* sequencing and MALDI-TOF MS), *rpoB* sequencing resulted in the most significant separation and therefore most consistent grouping (Global tests, Table 4). The global ANOSIM tests based on the 16S rRNA and MALDI-TOF matrices revealed only one significant test each (Table 4). Among the genomic fingerprinting methods, ERIC-PCR showed the most stable and significant grouping (Table 5).

TABLE 4. ANOSIM statistics (global and pairwise test) based on sequence (16S rRNA / *rpoB*, ARB) and MALDI-TOF (Ochiai) resemblance matrices. Comparison of *Vibrio* isolate grouping due to the investigation methods 16S rRNA (BLAST) / *rpoB* (ARB) sequencing, MALDI-TOF MS, detection of *tlh*, *toxR*, Kanagawa phenomenon. 999 permutations, significant values in bold.

	Global Test		**Pairwise Tests**		
	Sample Statistik (Global R)	**Significance level of sample statistic [%]**	**Groups**	**R Statistic**	**Significance level [%]**
16S rRNA (ARB)					
MALDI-TOF	0.025	31.3	only 2 groups (*V.p.*, *V.a.*)		
tlh	0.123	0.4	+, (+)	0.165	0.9
			+, -	0.193	0.4
			(+), -	-0.006	47.8
toxR	0.127	4.8	+, -	0.207	1.1
			+, (+)	0.376	0.3
			-, (+)	-0.062	74.1
Kanagawa	0.063	23.6	+, -	0.125	13.5
			+, (+)	0.076	21.5
			-, (+)	0.008	44.2
rpoB (ARB)	**0.454**	**0.1**	**I, IIa**	**0.671**	**0.1**
			I, IIb	**0.363**	**0.1**
			IIa, IIb	**0.476**	**0.1**
***rpoB* (ARB)**					
16S (BLAST)	0.112	5.6	*V.p.*, *V.* sp.	0.172	2.9
			V.p., *V.a.*	0.097	6.3
			V. sp., *V.a.*	0.215	5.3
MALDI-TOF	**0.398**	**0.1**	only 2 groups (*V.p.*, *V.a.*)		
tlh	**0.501**	**0.1**	**+, (+)**	**0.676**	**0.1**
			+, -	**0.66**	**0.1**
			(+), -	-0.031	70.6
toxR	**0.739**	**0.1**	**+, -**	**1**	**0.1**
			+, (+)	**1**	**0.1**
			-, (+)	0.059	22.1
Kanagawa	**0.698**	**0.1**	**+, -**	**0.872**	**0.1**
			+, (+)	0.247	6.1
			-, (+)	**0.632**	**0.1**
MALDI-TOF MS					
16S (BLAST)	0.015	42.3	*V.p.*, *V.* sp.	0.083	25
			V.p., *V.a.*	-0.009	49.9
			V. sp., *V.a.*	-0.097	80.5
tlh	**0.274**	**0.1**	**+, (+)**	**0.264**	**0.1**
			+, -	0.33	0.3
			(+), -	0.226	0.8
toxR	0.175	0.3	+, -	0.236	0.3
			+, (+)	0.181	2.9
			-, (+)	0.102	13.8
Kanagawa	0.068	19.7	+, -	0.189	2.9
			+, (+)	0.413	2
			-, (+)	-0.143	89
rpoB (ARB)	0.147	0.5	I, IIa	0.148	3.6
			I, IIb	0.25	0.3
			IIa, IIb	0.04	24.7

+: strong signal, -: no signal, (+): weak signal; *V.*: *Vibrio*, *a.*: *alginolyticus*, *p.*: *parahaemolyticus*, sp.: undefined species; I, IIa, IIb: ARB grouping according to *rpoB* sequencing

TABLE 5. ANOSIM statistics (global and pairwise test) based on sequence (16S rRNA / *rpoB*, ARB) and MALDI-TOF (Ochiai) resemblance matrices. Comparison of *Vibrio* isolate grouping due to the investigation methods 16S rRNA (BLAST) / *rpoB* (ARB) sequencing, MALDI-TOF MS, detection of *tlh*, *toxR*, Kanagawa phenomenon. 999 permutations, significant values in bold.

	Global Test		**Pairwise Tests**		
	Sample Statistik (Global R)	**Significance level of sample statistic [%]**	**Groups**	**R Statistic**	**Significance level [%]**
BOX-PCR					
16S (BLAST)	**0.288**	**0.1**	*V.p.*, *V.* sp.	0.247	2.3
			V.p., *V.a.*	0.347	0.2
			V. sp., *V.a.*	-0.082	71
MALDI-TOF	0.186	0.5	only 2 groups (*V.p.*, *V.a.*)		
tlh	**0.293**	**0.1**	**+, (+)**	**0.419**	**0.1**
			+, -	0.381	0.2
			(+), -	0.031	21.8
toxR	**0.35**	**0.1**	**+, -**	**0.474**	**0.1**
			+, (+)	**0.569**	**0.1**
			-, (+)	0.068	22.9
Kanagawa	0.28	0.2	+, -	0.358	0.3
			+, (+)	0.305	1.8
			-, (+)	0.214	7.2
rpoB (ARB)	**0.672**	**0.1**	**I, IIa**	**0.564**	**0.1**
			I, IIb	**0.777**	**0.1**
			IIa, IIb	**0.634**	**0.1**
ERIC-PCR					
16S (BLAST)	-0.017	57.1	*V.p.*, *V.* sp.	0.019	39.6
			V.p., *V.a.*	-0.006	50.1
			V. sp., *V.a.*	-0.096	77.7
MALDI-TOF	**0.305**	**0.1**	only 2 groups (*V.p.*, *V.a.*)		
tlh	**0.491**	**0.1**	**+, (+)**	**0.594**	**0.1**
			+, -	**0.682**	**0.1**
			(+), -	0.154	1.1
toxR	**0.57**	**0.1**	**+, -**	**0.824**	**0.1**
			+, (+)	**0.857**	**0.1**
			-, (+)	0.008	43.8
Kanagawa	**0.487**	**0.1**	**+, -**	**0.621**	**0.1**
			+, (+)	0.3	2.4
			-, (+)	0.369	1.2
rpoB (ARB)	**0.789**	**0.1**	**I, IIa**	**0.829**	**0.1**
			I, IIb	**0.941**	**0.1**
			IIa, IIb	**0.583**	**0.1**
REP-PCR					
16S (BLAST)	0.073	17.5	*V.p.*, *V.* sp.	-0.013	53.4
			V.p., *V.a.*	0.157	4.5
			V. sp., *V.a.*	0.014	42.3
MALDI-TOF	**0.267**	**0.1**	only 2 groups (*V.p.*, *V.a.*)		
tlh	**0.244**	**0.1**	**+, (+)**	**0.333**	**0.1**
			+, -	**0.294**	**0.1**
			(+), -	0.082	7
toxR	**0.366**	**0.1**	+, -	**0.507**	**0.1**
			+, (+)	0.585	0.2
			-, (+)	0.039	29
Kanagawa	0.33	0.2	**+, -**	**0.46**	**0.1**
			+, (+)	0.079	23.3
			-, (+)	0.192	8.1
rpoB (ARB)	**0.498**	**0.1**	**I, IIa**	**0.579**	**0.1**
			I, IIb	**0.655**	**0.1**
			IIa, IIb	**0.41**	**0.1**

+: strong signal, -: no signal, (+): weak signal; *V.*: *Vibrio*, *a.*: *alginolyticus*, *p.*: *parahaemolyticus*, sp.: undefined species; I, IIa, IIb: ARB grouping according to *rpoB* sequencing

Based on the BOX-PCR matrices, the isolate grouping was also significant in several cases, but the global R values ranged below the ones of the ERIC-PCR ANOSIM. The least distinct isolate grouping was achieved using REP-PCR (Table 5). To further assess the consistency of the groupings, pairwise tests were performed. Overall, the groups based on the method '16S BLAST' were the least stable. The most consistent groups were those based on the '*rpoB* ARB' and '*toxR*' assignment. Best separated were the groups '*toxR* +' and '*toxR* -' as well as '*toxR* +' and '*toxR* (+) ' when calculated based on the results of the *rpoB* sequencing (R value = 1). The separation based on ERIC-PCR was also highly consistent: Especially the '*toxR*' (+ / -, R= 0.824; + / (+), R= 0.857) and '*rpoB* ARB' (I / IIa, R= 0.829; I / IIb, R= 0.941) grouping was highly significant. Ordination of the Jaccard similarities of the ERIC fingerprint data was carried out by MDS (Figure 3). The MDS illustrates that, due to the ERIC fingerprint patterns, the isolates fell into three distinct clusters which is in perfect accordance to the *rpoB* classification. The DSM 2171 and 2172 strains constituted the sole exceptions. All *Vibrio* isolates in the first similarity cluster were *toxR* and *tlh* positive and also (slightly) positive for the Kanagawa phenomenon. Only strain JF57 was Kanagawa negative. In the other clusters, no isolate was positive for *toxR* or strongly positive for the Kanagawa phenomenon and only few isolates were *tlh* positive.

Moreover, the similarity matrices of the genomic fingerprint data, the 16S rRNA / *rpoB* sequencing and the MALDI-TOF MS analysis were compared using the 2STAGE and RELATE tools of PRIMER. The relations between the methods are illustrated in a MDS diagram using Spearman correlations (Figure 4). The comparison of the similarity matrices revealed that the results of the *rpoB* sequencing and the fingerprint method ERIC-PCR correlated the most with an R value of 0.74. Correlation of the results of *rpoB* sequencing and the other rep-PCRs BOX and REP was also significant, with R values between 0.4 and 0.5. Even though the 16S rRNA sequencing method was more distant to the other methods in the MDS, it correlated significantly with the results of the BOX- / REP-PCR and *rpoB* sequencing (R values below 0.4). The results of the MALDI-TOF MS analysis significantly correlated only with the results of the ERIC-PCR (R = 0.16). The low similarity of the MALDI-TOF data with the data of the other methods is illustrated by the outlying position of MALDI-TOF in the MDS plot (Figure 4).

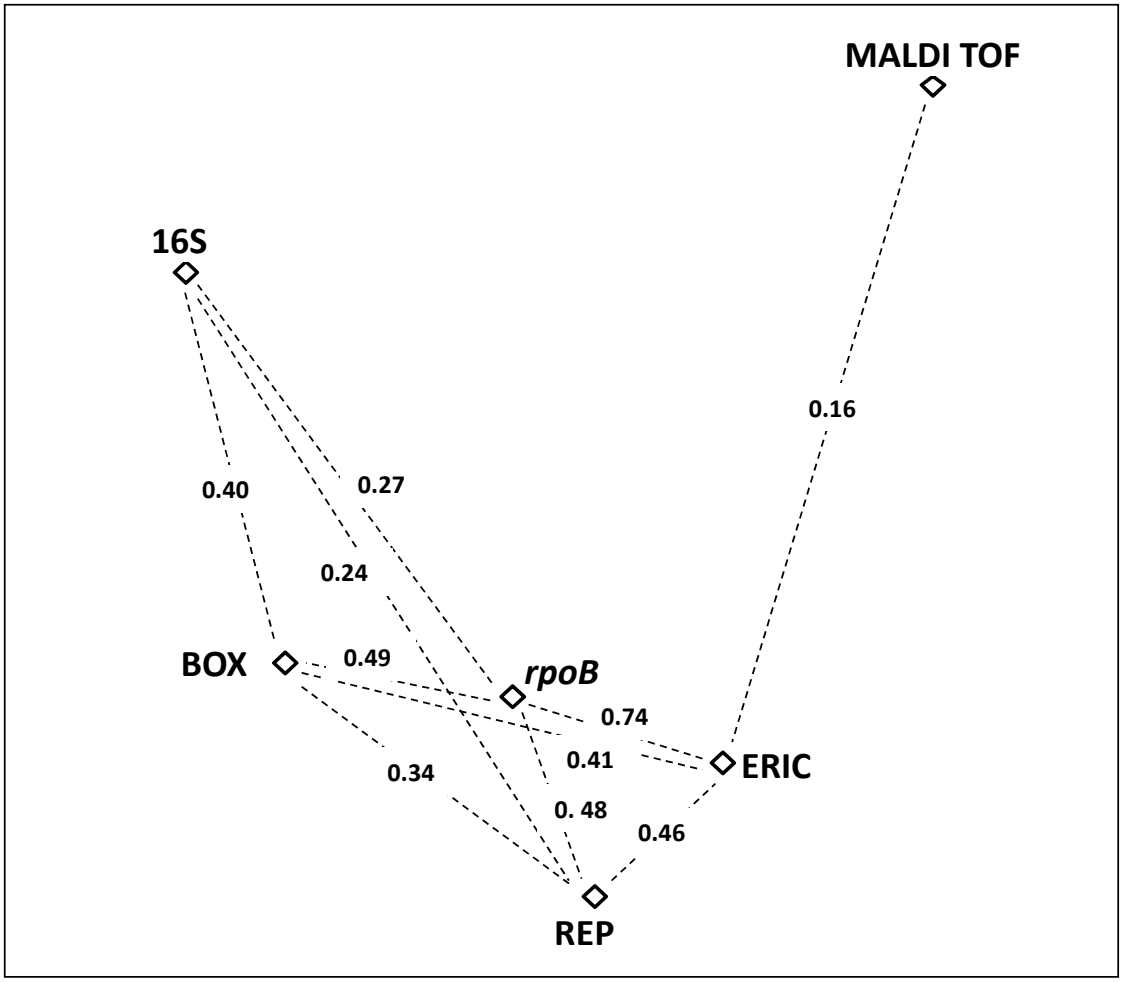

FIGURE 4. MDS plot for Spearman correlations of the results of different classification methods (BOX-, ERIC-, REP-PCR, 16S rRNA / *rpoB* sequencing, MALDI-TOF). Similarity matrices of the results were compared using the PRIMER subroutines 2STAGE and RELATE. 2D stress value is 0.03. Method names are connected, when results of the methods correlate significantly. R values are given.

Discussion

In this study we investigated potential human pathogenic *Vibrio* spp. in the German Bight, with particular focus on *V. parahaemolyticus*. The *Vibrio* strains isolated at Helgoland Roads were generally assigned to the species *V. harveyi, V. parahaemolyticus* and *V. alginolyticus*. But due to our focus, we omitted the *V. harveyi* isolates from our analyses. It is already known from the literature that *V. parahaemolyticus* and *V. alginolyticus* are hard to distinguish due to their close phylogenetic relationship (Kita-Tsukamoto*, et al.*, 1993, Robert-Pillot*, et al.*, 2002). Nevertheless, a number of isolates could be clearly identified as the potentially pathogenic species *V. parahaemolyticus*, when the results of all applied classification methods were taken into account (MALDI-TOF MS, 16S rRNA / *rpoB* sequencing; Table 2). However, when identification is restricted to phylogenetic classification of the 16S rRNA gene sequence, the species *V. parahaemolyticus* and *V. alginolyticus* are indistinguishable (Figure 1). In contrast, using *rpoB* sequencing, the resolution of the *Vibrio* spp. in the phylogenetic tree was much more pronounced (Figure 2). The groups were more distinct and a clear separation between *V. parahaemolyticus* ('*Vibrio* group I (*rpoB*)') and *V. alginolyticus* ('*Vibrio* groups IIa / IIb (*rpoB*)') was achieved. This is consistent with other studies, such as the one of Ki *et al.* (2009). ANOSIM analysis revealed that the *rpoB*-based isolate classification was highly significant and consistent (Tables 4 and 5). Though the *rpoB* sequence-based classification and MALDI-TOF results agreed in over 80% of the isolates, statistical analysis of the MALDI-TOF MS results indicated this method to result in the least consistent grouping. Prior studies showed that MALDI-TOF MS is a very fast and accurate tool for the identification and classification of bacterial isolates (Fenselau & Demirev, Mellmann*, et al.*, 2008, Sauer*, et al.*, 2008). However, this is one of the first studies using MALDI-TOF MS to classify environmental *Vibrio* isolates. Besides its great potential to serve as fast *Vibrio* classification tool we revealed also some need for improvement concerning MALDI-TOF MS. To enhance the reliability of this method for the differentiation of closely related *Vibrio* species, such as *V. parahaemolyticus* and *V. alginolyticus*, the MALDI Biotyper database must be extended to include more environmental isolates, such as those ones obtained in this study. The databases used for BLAST (GenBank; >100,000,000 sequences) and ARB (>100,000 sequences) currently contain far more information than the database for the MALDI Biotyper software (>3000 reference

spectra of 1900 species). But it could been demonstrated so far that MALDI-TOF MS can be used as robust method to determine the *Vibrio* genus, or even to differentiate between distantly related species, such as *V. parahaemolyticus* and *V. harveyi*. MALDI-TOF MS is a highly time-saving and labor-effective alternative to other initial classification methods.

In addition to sequence and MALDI-TOF MS analysis, we targeted species-specific genes, *tlh* and *toxR*, to identify *V. parahaemolyticus* isolates. PCR especially targeting the *toxR* gene is described as a highly reliable tool for the detection of *V. parahaemolyticus* (Kim*, et al.*, 1999, Rosec*, et al.*, 2009). The *rpoB*-based assignment of *V. parahaemolyticus* was in complete agreement with the presence of *toxR* (R = 1; ANOSIM Table 4), diagnostic of this species. The MDS ordination based on the ERIC fingerprint pattern (Figure 3) illustrates that all *toxR*-positive isolates belong to the same cluster that harbored all isolates from '*Vibrio* group I (*rpoB*)'. It can be assumed that all isolates in '*Vibrio* group I (*rpoB*)' represent *V. parahaemolyticus* strains. The three *tlh*-positive isolates, which were only slightly positive for *toxR*, were identified as *V. alginolyticus* by *rpoB* sequencing. This suggests that a slight signal for *toxR* does not indicate *V. parahaemolyticus*, and should rather be interpreted as *toxR* negative. The ANOSIM calculations corroborate this assumption. The differences between groups '*toxR* +' and '*toxR* –' and '*toxR* +' and '*toxR* (+)' were significant, but the separation between '*toxR* –' and '*toxR* (+)' was inconsistent (Tables 4 and 5). This was also the case for slightly positive *tlh* signals. Overall, this study indicates a higher species specificity of the *toxR*- than of the *tlh*-targeting PCR. This is in agreement with a comparable study by Croci *et al.* (2007a).

We also tested the *Vibrio* strains for the Kanagawa phenomenon, a physiological indicator for pathogenicity. The Kanagawa phenomenon is indicated by characteristic halos on Wagatsuma blood agar, which implies the ability of bacteria to hemolyse human erythrocytes. Hemolytic ability of *Vibrio* bacteria is attributed to the presence of the *tdh* gene (Nishibuchi & Kaper, 1995, Okuda & Nishibuchi, 1998). However, though *tdh* was not detected in any of the *Vibrio* isolates from Helgoland Roads, seven isolates were clearly positive for the Kanagawa phenomenon (Table 3). This pattern has been reported previously by Bej *et al.* (Bej*, et al.*, 1999), suggesting that factors other than the presence of the *tdh* gene are involved in the haemolytic ability of *V. parahaemolyticus* (Izutsu*, et al.*, 2008, Caburlotto*, et al.*, 2009). As illustrated by MDS ordination in our study (Figure 3), most *toxR* positive isolates were at least

slightly able to hemolyse blood. As such, one should not underestimate the potential hazard posed by environmental *V. parahaemolyticus* strains lacking *tdh* and *trh*. In 2009, Garcia *et al.* described the risk of non-pandemic *tdh*- and *trh*-negative *V. parahaemolyticus* strains, which coexist with pandemic strain. Moreover, the study of Garcia *et al.* (2009) postulated the vast dynamics of *V. parahaemolyticus* communities. The marine environment has a high potential to serve as a reservoir for pathogenicity factors such as *tdh* and *trh* (Xie*, et al.*, 2005, Ellingsen*, et al.*, 2008, Stewart*, et al.*, 2008). Under given environmental conditions, such as rising water temperatures, pathogenic serovars may spread further and transfer pathogenicity factors to formerly non-pathogenic strains. A warming trend could already been detected in the North Sea (Wiltshire & Manly, 2004), which -in addition to the comparably low salinity in this region- makes this ecosystem more attractive for potential pathogens such as *V. parahaemolyticus.* In this context, a recent review of Baker-Austin *et al*. (2010) emphasizes the importance for monitoring and further research in European waters. The last step in our hierarchical approach was the genomic fingerprinting using BOX-, ERIC and REP-PCR to investigate the intraspecific diversity of the *V. parahaemolyticus* and *V. alginolyticus* strains at Helgoland Roads. Comparing the three rep-PCR methods, ERIC-PCR revealed the clearest results concerning similarity structures and discriminatory power. The fingerprint pattern revealed important insights into the *V. parahaemolyticus* / *V. alginolyticus* community at Helgoland Roads, which is obviously shaped by three distinct clusters (Figure 3). Combined with the results of the *rpoB* sequencing and the detection of *toxR*, we conclude that one cluster consisted exclusively of the potentially pathogenic species *V. parahaemolyticus*. Most likely, the other two clusters consisted of *V. alginolyticus* isolates or, in light of the slightly positive *toxR*, *tlh*, and hemolysis reactions, of one *V. alginolyticus* group and one hybrid group of both species. Mutation events and lateral gene transfer are frequent within the genus *Vibrio* and lead to high intraspecies variability and polymorphism of environmental strains (Moreno, *et al*., 2002, Gonzales-Escalona, *et al*., 2005, Hazen, *et al*., 2009a). It is thus likely that several Helgoland *Vibrio* isolates represent very close relatives, or even species hybrids.

Thus far, we could not detect any serious human pathogenic *Vibrio* strains in the German Bight. However, as many strains of the potential pathogen *V. parahaemolyticus* were identified, there is a great urgency for the definition of standardized detection methods and further monitoring. In a previous study *V. alginolyticus* was found to be

the dominant mesophilic *Vibrio* species in the German Bight (Lhafi & Kühne, 2007). However, in the light of this study, it cannot be excluded, that the occurrence of *V. parahaemolyticus* might be underestimated in previous studies if only few or improper identification methods are applied.

This study develops and validates a multi-level approach to accurately assess *Vibrio* communities by: (1) MALDI-TOF MS analysis for an initial classification, (2) *rpoB* sequencing for the further *Vibrio* species identification, (3) detection of *toxR* to confirm *V. parahaemolyticus* strains, (4) screening for *tdh*/*trh* as pathogenicity indicator and (5) performing ERIC-PCR fingerprinting for insights into the diversity of *Vibrio* strains. The finding of the potentially pathogenic species in the German Bight requires ongoing investigations. It is crucial to monitor emerging pathogens in the North Sea as this ecosystem, which many of European industries rely on, experiences the effects of global climate change.

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CHAPTER II

Occurrence of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* in the German Bight over a seasonal cycle

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Abstract

Bacteria of the genus *Vibrio* are an important component of marine ecosystems worldwide. The genus harbors several human pathogens, for instance the species *Vibrio parahaemolyticus*, a main cause for foodborne gastroenteritis in Asia and the USA. Pathogenic *V. parahaemolyticus* strains emerged also in Europe, but little is known about the abundance, pathogenicity and ecology of *V. parahaemolyticus* in European waters. This study focuses on *V. parahaemolyticus* and its close relative *V. alginolyticus* in the North Sea (Helgoland Roads, Germany). Free-living, plankton-attached and shellfish-associated *Vibrio* spp. were quantified between May 2008 and January 2010. CFUs up to 4.3 x 10^3 N x L^{-1} and MPNs up to 240 N x g^{-1} were determined. Phylogenetic classification based on *rpoB* gene sequencing revealed *V. alginolyticus* as the dominant *Vibrio* species at Helgoland Roads, followed by *V. parahaemolyticus*. We investigated the intraspecific diversity of *V. parahaemolyticus* and *V. alginolyticus* using ERIC-PCR. The fingerprinting disclosed three distinct groups at Helgoland Roads, representing *V. parahaemolyticus*, *V. alginolyticus* and one group in between. The species *V. parahaemolyticus* occurred mainly in summer months. None of the strains carried the virulence-associated genes *tdh* or *trh*. We further analyzed the influence of nutrients, secchi depth, temperature, salinity, chlorophyll a and phytoplankton on the abundance of *Vibrio* spp. and the population structure of *V. parahaemolyticus*. Spearman Rank analysis revealed that particularly temperature correlated significantly with *Vibrio* spp. numbers. Based on multivariate statistical analyses we report that the *V. parahaemolyticus* population was structured by a complex combination of environmental parameters. To further investigate these influences is the key to understand the dynamics of *V. parahaemolyticus* in temperate European waters, where this pathogen is likely to gain in importance.

Introduction

Bacteria of the genus *Vibrio* represent an important component of the marine bacterioplankton and occur in free-living or plankton-attached forms. Some *Vibrio* species are subject to particular research attention, as they are pathogenic to humans. For instance, *V. parahaemolyticus* is the leading cause of human gastroenteritis in America and Asia (Su & Liu, 2007).

In recent years, pathogenic *V. parahaemolyticus* strains emerged also in temperate European waters (Martinez-Urtaza, *et al.*, 2005, Baker-Austin, *et al.*, 2010). Further spreading of this pathogen might be expected due to climate change, since *V. parahaemolyticus* is known to favor warmer water temperatures (Sobrinho, *et al.*, 2010). In addition to temperature, salinity appears to have a strong influence on the abundance of *V. parahaemolyticus* (Martinez-Urtaza, *et al.*, 2008b). Nevertheless, investigations on the influence of environmental parameters on populations of pathogenic *Vibrio* species in Northern European waters are rare. Little is known on the dynamics of the *Vibrio* community in these temperate waters and a limited number of studies address the *Vibrio* community in the North Sea (Bauer, *et al.*, 2006, Lhafi & Kühne, 2007, Ellingsen, *et al.*, 2008, Schets, *et al.*, 2010).

In a previous study, a polyphasic approach for the analysis of *V. parahaemolyticus* and *V. alginolyticus* strains from temperate waters was suggested (Oberbeckmann, *et al.*, 2011). This analysis scheme includes (i) sequencing of the *rpoB* gene (encoding the RNA polymerase β-subunit) to differentiate even closely related *Vibrio* species (Mollet, *et al.*, 1997, Tarr, *et al.*, 2007, Ki, *et al.*, 2009), (ii) use of *toxR* gene as a specific marker for the potential pathogen *V. parahaemolyticus* (Lin, *et al.*, 1993, Kim, *et al.*, 1999, Bauer & Rørvik, 2007), (iii) screening for virulence-associated genes, *tdh* and *trh* (Nishibuchi & Kaper, 1985, Honda, *et al.*, 1989, Nishibuchi, *et al.*, 1989, Tada, *et al.*, 1992), and (iv) repetitive sequences using ERIC fingerprinting (Hulton, *et al.*, 1991, Versalovic, *et al.*, 1991) to identify the intraspecies variability of *V. parahaemolyticus* and *V. alginolyticus* at Helgoland Roads.

We applied this hierarchical analysis scheme in this study, combined with statistical analyses, to investigate the *Vibrio* community at Helgoland Roads (North Sea, Germany) by determining its abundance, composition and response to environmental parameters. Spearman rank analyses and multiple regression calculations were performed to display the influence of environmental parameters (namely, temperature,

salinity, secchi depth, nutrient concentrations and phytoplankton density) on the *Vibrio* spp. abundance. Multivariate statistical analysis, e.g. analysis of similarity (ANOSIM) or correspondence analysis (CA), was applied to estimate the influence of environmental parameters on the structure of the *Vibrio* community, as well as specific populations, as special emphasis was given to the pathogen *V. parahaemolyticus* and its close relative *V. alginolyticus*.

We set out to ask: (1) How abundant are mesophilic *Vibrio* spp. at Helgoland Roads? (2) What *V. parahaemolyticus* / *V. alginolyticus* populations do we find at Helgoland Roads? (3) Which environmental parameters affect this population through the year? (4) Can we use these observations to make predictions concerning the pathogenic potential of *Vibrio* communities in temperate waters?

Material and Methods

Sample collection

Seawater samples were taken biweekly using the RV Aade at Helgoland Roads (North Sea, Germany, 54°11.3 ' N, 7°54.0 ' E) from May 2008 to January 2010. Additionally, net haul samples (> 20 µm and > 150 µm) were taken from March 2009 to January 2010 at the same location. Specimens of *Mytilus edulis* (mussels) were collected monthly in the South Harbor of the island of Helgoland (54°10.5 ' N; 7°53.67 ' E) from May to November 2009. All samples were analyzed directly. Concurrently, nutrients (SiO_2, PO_4^{3-}, NO_2, NO_3^-, NH_4^+), secchi depth, temperature, salinity and phytoplankton abundance (diatoms, dinoflagellates) data were measured (Table 1).

TABLE 1. List of measured environmental parameters, abbreviations, units and measurement methods / instruments.

Parameter	Abbreviation	Unit	Measurement
Secchi depth	Secchi	m	Secchi disk (directly on board ship)
Temperature	T	°C	Thermometer (directly on board ship)
Salinity	S		Salinometer Autosal, Guideline
SiO_2		µmol x L^{-1}	Chemical / photometric determination (Grasshoff *et al.*, 1999)
PO_4^{3-}		µmol x L^{-1}	
NO_2		µmol x L^{-1}	
NO_3^-		µmol x L^{-1}	
$NH4^+$		µmol x L^{-1}	
Diatoms		N x L^{-1}	Utermöhl counting (Utermöhl, 1958) Inverted microscope Axiovert 135
Dinoflagellates	Dino	N x L^{-1}	

Sample preparation, *Vibrio* spp. quantification and cultivation

Unfiltered seawater and net haul samples (> 20 µm and > 150 µm) were investigated on a biweekly basis, for the quantification, cultivation and phylogenetic classification of *Vibrio* isolates. The seawater sample was filtered (10 µm) and 100, 50 and 10 ml of the filtrate were concentrated on 0.2 µm membrane filters in three replicates. For simplicity, this fraction is referred to as the free-living bacteria fraction ('> 0.2 µm'). The 20 µm net haul sample was concentrated on 20 µm gauze to investigate *Vibrio* spp. attached to plankton greater than 20 µm ('>20 µm'). The 150 µm net haul sample was concentrated on 100 µm gauze to investigate *Vibrio* spp. attached to plankton greater than 100 µm ('>100 µm'). Both net haul enrichments were homogenized in sterile seawater using an Ultra-Turrax® (Janke & Kunkel; Staufen, Germany). Volumes of 10, 5 and 0.3 ml of the

homogenates were concentrated on 0.2 µm membrane filters in three replicates. All membrane filters were placed on TCBS agar plates (Kobayashi*, et al.*, 1963) and incubated at 37°C for 24 h to selectively cultivate mesophilic and potentially pathogenic *Vibrio* spp.. All green and yellow colonies were counted after 24 h and the CFU (colony forming units) were determined according to the German DIN standard method (Feuerpfeil*, et al.*, 2002). Starting in March 2009, single green or yellow colonies were further transferred to 50% seawater marine broth agar and serially re-inoculated to pure cultures. In addition to free-living and plankton-attached *Vibrio* spp., shellfish-associated *Vibrio* spp. from Helgoland Roads were investigated monthly. 25 g *M. edulis* tissue was homogenized in 225 ml Alkaline Peptone Water (APW) using an Ultra-Turrax®. To prepare the first dilution, 1 mL of the homogenate was mixed with 9 mL APW. Subsequent dilution of the homogenate, transfer to TCBS agar and evaluation of the shellfish-associated *Vibrio* spp. was carried out using a standard MPN (most probable number) protocol (Jark & Kirschke, 2009). The cultivation of *Vibrio* spp. was carried out on TCBS as described above for seawater/plankton samples.

Identification of *Vibrio* spp. isolates

To confirm the affiliation to the genus *Vibrio*, all isolates were screened with the specific oligonucleotide probe GV (Table 2) by fluorescence *in situ* hybridization (FISH) (Giuliano*, et al.*, 1999, Eilers*, et al.*, 2000). From cultures on solid medium one inoculation loop was suspended in sterile seawater and the cells were fixed with PFA (paraformaldehyde, final concentration 4%). An aliquot of 20 µl of each suspension was placed on a multi-well glass slide, dried, dehydrated (ethanol 50, 80 and 96%) and subsequently hybridized according to Pernthaler *et al.* (Pernthaler, 2001). The final formamide concentration in the hybridization buffer (900 mM NaCl, 20 mM Tris/HCl, 0.01 % SDS) was 30% and the final NaCl concentration in the washing buffer (20 mM Tris/HCl, 5 mM EDTA, 0.01 % SDS) was 100 mM. The visual inspection was performed by epifluorescence microscopy (Zeiss). We used the strain *V. parahaemolyticus* RIMD 2210633 (kindly provided by Carsten Matz, HZI) as positive control and the strain *Bacillus licheniformis* SO30 (in-house culture collection) as negative control. GV-positive isolates were assigned to the genus *Vibrio*.

DNA extraction

The genomic DNA of all *Vibrio* isolates was extracted using lysozyme/SDS lysis and phenol/chloroform extraction, followed by isopropanol precipitation, as described previously (Sapp*, et al.*, 2007).

RpoB gene sequencing

In this study, *rpoB* gene sequencing was performed, due to its power to differentiate closely related *Vibrio* species at a higher resolution than the 16S rRNA gene (Ki*, et al.*, 2009, Oberbeckmann*, et al.*, 2011). Approximately 1600 bp of the *rpoB* fragment was amplified with primers rpoB458F, rpoB2105R and rpoB1110F (Table 2), according to Tarr *et al.* (2007) and Hazen *et al.* (2009a). Sequencing of *rpoB* was carried out using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA). The resulting sequences were aligned using AlignIR1.2® (LI-COR).

TABLE 2. List of applied primer sets and probe, primer / probe sequences, target genes, fragment sizes and references.

Primer/Probe	Sequence (5´-3´)	Target gene	Length	Reference
rpoB458F	agg cgt gtt ctt cga cag cga taa	*rpoB*	1600	Hazen et al., 2009a
rpoB2105R	cgg cta cgt tac gtt cga tac cag			
rpoB1110F	gta gaa atc tac cgc atg atg			Tarr et al., 2007
UtoxF	gas ttt gtt tgg cgy gar caa ggt t	*toxR*	300	Bauer & Rørvik, 2007
vptoxR	ggt tca acg att gcg tca gaa g			
tdh-R	tgg aat aga acc ttc atc ttc acc	*tdh*	270	Nishibuchi & Kaper, 1985
tdh-L	gta aag gtc tct gac ttt tgg ac			
trh-R	cat aac aaa cat atg ccc att tcc g	*trh*	500	Honda & Iida, 1993; Honda et al., 1991
trh-L	ttg gct tcg ata ttt tca gta tct			
ERIC2	aag taa gtg act ggg gtg agc g		diverse	Versalovic et al., 1991
ERIC1R	atg taa gct cct ggg gat tca c			
		position 841 - 860 of 16S rRNA		
GV	agg cca caa cct cca agt ag	*Vibrio* spp.	20	Eilers et al., 2000; Giuliano et al., 1999

Phylogenetic analysis

The *rpoB* gene sequences were analyzed phylogenetically using the ARB® software package (Ludwig*, et al.*, 2004) and a custom *rpoB* database containing all *rpoB* sequences available in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/). After the addition of our sequences to the ARB *rpoB* database, the sequence alignment was carried out with the integrated "Fast Aligner". The 1300-1600 bp long sequences were used to calculate the phylogenetic tree. The phylogenetic relationships were deduced by the neighbour-joining method with Felsenstein correction (Felsenstein, 1993).

The sequences obtained in this study are available from GenBank under the accession numbers HQ533880 - HQ534045.

PCR analysis of regulatory and virulence-related genes

Specific PCR for *toxR* (Bauer & Rørvik, 2007), *tdh* (Nishibuchi & Kaper, 1985) and *trh* (Honda*, et al.*, 1991, Honda & Iida, 1993) genes was performed with DNA extracts from the isolates identified as *V. parahaemolyticus* according to *rpoB* sequencing (applied primer: see Table 2). The DNA of the strain *V. parahaemolyticus* RIMD 2210633 served as reference, as this strain is known to harbor *toxR* and *tdh*. *V. parahaemolyticus* CM24 (kindly provided by Carsten Matz, HZI) was used as a positive control for the PCR reaction targeting *trh*. All reactions were performed in duplicate. In the case of discordant results, a third PCR was carried out. The PCR reactions and the subsequent verification of the products were performed as described previously (Oberbeckmann, *et al*., 2011).

Genomic fingerprinting using ERIC-PCR

The genomic fingerprints of the isolates belonging to the *V. parahaemolyticus* and *V. alginolyticus* I / II groups - according to the *rpoB* gene sequencing - were performed in triplicates using ERIC-PCR, as described previously (Oberbeckmann*, et al.*, 2011). A triplicate per isolate built a composite pattern for the evaluation and comparative analysis with the BioNumerics 5.10 software (Applied Maths, Belgium). To normalize the gel images, a 1 kb ladder served as reference. Bands were searched automatically by the software (settings: 5% minimum profiling, relative to maximal value) and adjusted manually. In order to compare the band patterns of the isolates, band-matching analysis was performed. Based on their position, bands were assigned to classes and a band-matching table was created (settings: 1% optimization and position tolerances).

Statistical analysis

To investigate correlations between water transparency (as 'secchi depth'), temperature, salinity, SiO_2, PO_4^{3-}, NO_2, NO_3^-, NH_4^+, diatoms, dinoflagellates and the abundance of *Vibrio* spp., Spearman rank correlation analysis was carried out using the software STATISTICA (StatSoft, version 7.1). Missing values were pairwise excluded and the significance level for correlating variables was set to $p < 0.05$. A multiple regression was calculated using the SigmaPlot (version 11.0) to analyze the additive influence of

multiple factors on *Vibrio* abundances in the waters of Helgoland. To visualize the structure of the *V. parahaemolyticus / alginolyticus* population identified by ERIC fingerprinting, multivariate statistical analysis was performed with the Primer v6 software suite (PRIMER-E, Ltd., UK) (Clarke & Gorley, 2006) based on a similarity matrix of the ERIC-PCR band classes (Jaccard index). The results were grouped according to *rpoB* gene sequencing. Analysis of similarity (ANOSIM) was performed to test the hypothesis that the similarity within the resulting groups was greater than between the groups ($p = 0.1$). Ordination of the similarities of all *V. parahaemolyticus* and *V. alginolytiucs* isolates was carried out by multi-dimensional scaling (MDS). Due to clearness, the plot is presented 2-dimensional. The CANOCO statistical package for Windows 4.53 (Biometris) was applied to estimate the influence of environmental parameters on the structure of the individual *rpoB* groups *V. parahaemolyticus* and *V. alginolyticus* I / II. Correspondence analysis (CA) was carried out for each selected data subset (*rpoB* based grouping). The ordination of the *Vibrio* isolates was illustrated using a bi-plot scaling, in which the influence of the environmental parameters was indicated with arrows.

Results

Quantification of *Vibrio* spp. at Helgoland Roads

The *Vibrio* spp. from Helgoland Roads were quantified between May 2008 and January 2010 (Figure 1). Free-living *Vibrio* spp. ('> 0.2 µm') peaked three times within this period: 4.3 x 10^3 (August 08), 3.1 (December 08) and 2.1 (August 09) N x L^{-1}. No *Vibrio* spp. could be cultivated on certain dates in May and October 2008, as well as between February and May 2009. The '> 20 µm'- and '> 100 µm'-attached *Vibrio* spp. were quantified from March 2009 on. The abundances of the attached *Vibrio* spp. followed the same trend as the free-living *Vibrio* spp. and ranged between 0 and 45 N x L^{-1}, with the highest abundances in August (45 N x L^{-1} '> 20 µm'; 6 N x L^{-1} '> 100 µm'). The data for the attached *Vibrio* spp. are indicated per volume seawater instead of per weight plankton, because this study aims to detect seasonal trends of *Vibrio* abundances comparing different water fractions with each other. The MPN values for mesophilic *Vibrio* spp. in *M. edulis* tissue between May and November 2009 ranged between 0 and 240 MPN x g^{-1}, with a peak in July.

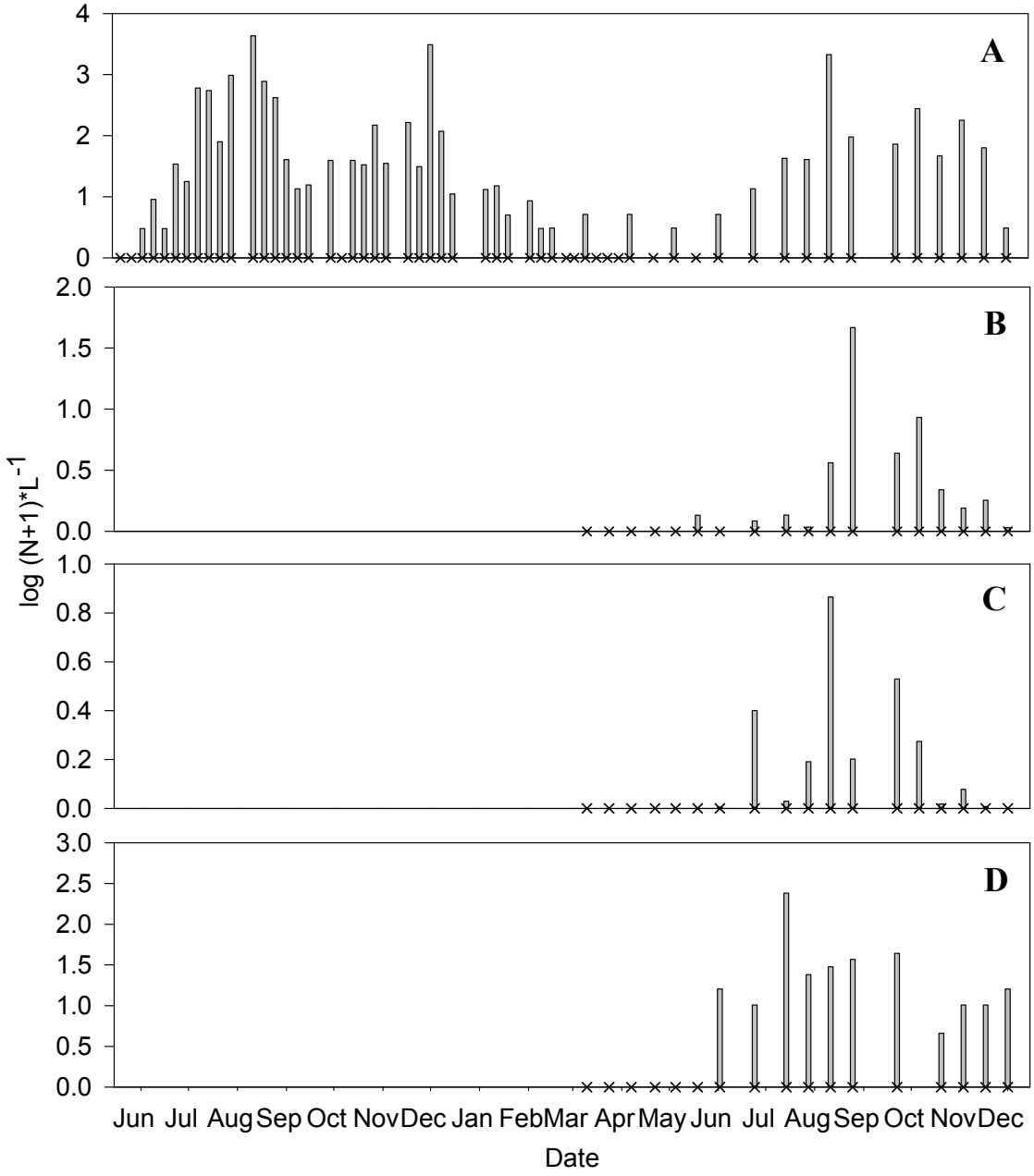

FIGURE 1. Log-transformed abundances of free-living (A), >20 µm-attached (B), >100 µm-attached (C) and *M edulis*-associated (D) *Vibrio* spp. between May 2008 and December 2009. Crosses indicate sampling days.

Environmental parameters

Between May 2008 and January 2010, data for temperature, salinity, nutrients (SiO_2, PO_4^{3-}, NO_2, NO_3^-, NH_4^+), secchi depth, diatom and dinoflagellate abundances were recorded. Temperature and diatom abundances were highest in August 2008 and 2009. Dinoflagellate abundances showed peaks in spring and summer of both years. Lowest temperatures could be detected between December 2008 and April 2009, lowest phytoplankton abundances were found between October 2008 and March 2009. Salinity

was highest between January and March 2009 and showed several drops and peaks within the recorded period (Figure 2C). The concentrations of dissolved inorganic nitrogen were highest in July, August and November 2008 and January and March 2009. PO_4^{3-} showed peaks in June, August and October 2008 and March and August to October 2009. Highest SiO_2 concentrations occurred in August and September 2008 and in January, March and September 2009. In general, the lowest nutrient concentrations were detected in early June and late September 2008 and June and July 2009. The Spearman correlations between the environmental parameters and the abundance of culturable *Vibrio* spp. are given in Table 3 with significant correlations indicated in bold characters. The free-living ('> 0.2 µm'), plankton-attached ('> 20 µm', '> 100 µm') and *M. edulis* associated *Vibrio* spp. all correlated significantly (< 0.05) with temperature (up to R=0.901), SiO_2 (up to R=0.801), NO_2 (up to R= - 0.890) and NO_3^- (up to R= - 0.825). Furthermore, the '> 20 µm'-attached *Vibrio* spp. correlated significantly with the parameter secchi depth, the '> 100 µm'-attached *Vibrio* spp. with PO_4^{3-}. *Vibrio* spp. from all fractions were positively correlated with each other. For the environmental variables, especially the algal groups correlated with many other parameters. Significant correlations of the diatoms and dinoflagellates with eight out of nine other parameters could be detected. To display the additive influence of multiple environmental parameters on the abundance of free-living *Vibrio* spp. at Helgoland Roads, a multiple regression model was calculated with all measured environmental parameters. The model was correlated significantly ($p < 0.0001$) with the observed abundances (Figure 2A). The observed values were distinctly higher or lower than the predicted ones only at certain periods, which is illustrated in the residual analysis (Figure 2B).

TABLE 3. Spearman Rank correlations for free-living ('>0.2 µm'), plankton-attached ('>20 µm', '>100 µm'), shellfish-associated *Vibrio* spp. and environmental parameters. Significant correlations ($p < 0.05$) indicated in bold.

											Vibrio spp.		
		Secchi	Temperature	Salinity	SiO_2	$PO_4^{3?}$	NO_2	NO_3^-	NH_4^+	Diatoms	Dinoflagellates	Free-living	Phytoplankton	Zooplankton
Vibrio spp.	Free-living	-0.187	**0.656**	-0.126	**0.475**	0.368	**-0.440**	**-0.599**	0.009	-0.007	0.198			
	Phytoplankton	**-0.611**	**0.758**	0.022	**0.801**	0.673	**-0.760**	**-0.825**	-0.084	0.086	0.006	**0.848**		
	Zooplankton	-0.342	**0.901**	-0.267	**0.633**	**0.445**	**-0.869**	**-0.818**	-0.244	0.184	0.294	**0.851**	**0.762**	
	Mytilus edulis	-0.118	**0.861**	-0.133	**0.645**	0.382	**-0.890**	**-0.783**	-0.048	-0.229	0.386	**0.685**	**0.627**	**0.779**
	Temperature	0.023												
	Salinity	0.055	**-0.346**											
	SiO_2	**-0.421**	0.182	0.009										
	$PO_4^{3?}$	**-0.506**	0.074	0.208	**0.727**									
	NO_2	-0.055	**-0.768**	0.173	0.194	0.226								
	NO_3^-	-0.046	**-0.823**	-0.106	-0.104	-0.110	**0.833**							
	NH_4^+	-0.247	**-0.297**	-0.090	**0.319**	0.252	**0.296**	**0.332**						
	Diatoms	0.149	**0.449**	**-0.404**	**-0.400**	**-0.322**	**-0.428**	**-0.319**	**-0.430**					
	Dinoflagellates	**0.333**	**0.537**	**-0.549**	**-0.340**	**-0.498**	**-0.541**	**-0.335**	-0.181	**0.562**				

Identification of *Vibrio* spp. isolates using FISH

Out of 277 tested isolates from TCBS agar, 166 (60%) could be identified as *Vibrio* spp. using the specific FISH probe GV. From the 166 *Vibrio* isolates, 51 isolates (30%) originated from the fraction '> 0.2 µm', 33 (20%) from the fraction '> 20 µm', 41 (25%) from the fraction '> 100 µm' and 41 (25%) from *M. edulis*.

Analysis of *rpoB* gene sequences

For more detailed information about the phylogenetic classification of the isolates, approximately 1600 bp of the *rpoB* genes of the 166 *Vibrio* isolates from Helgoland Roads were sequenced and analyzed using ARB. A neighbor-joining tree was created including closely related *rpoB* sequences from GenBank (Figure 3).

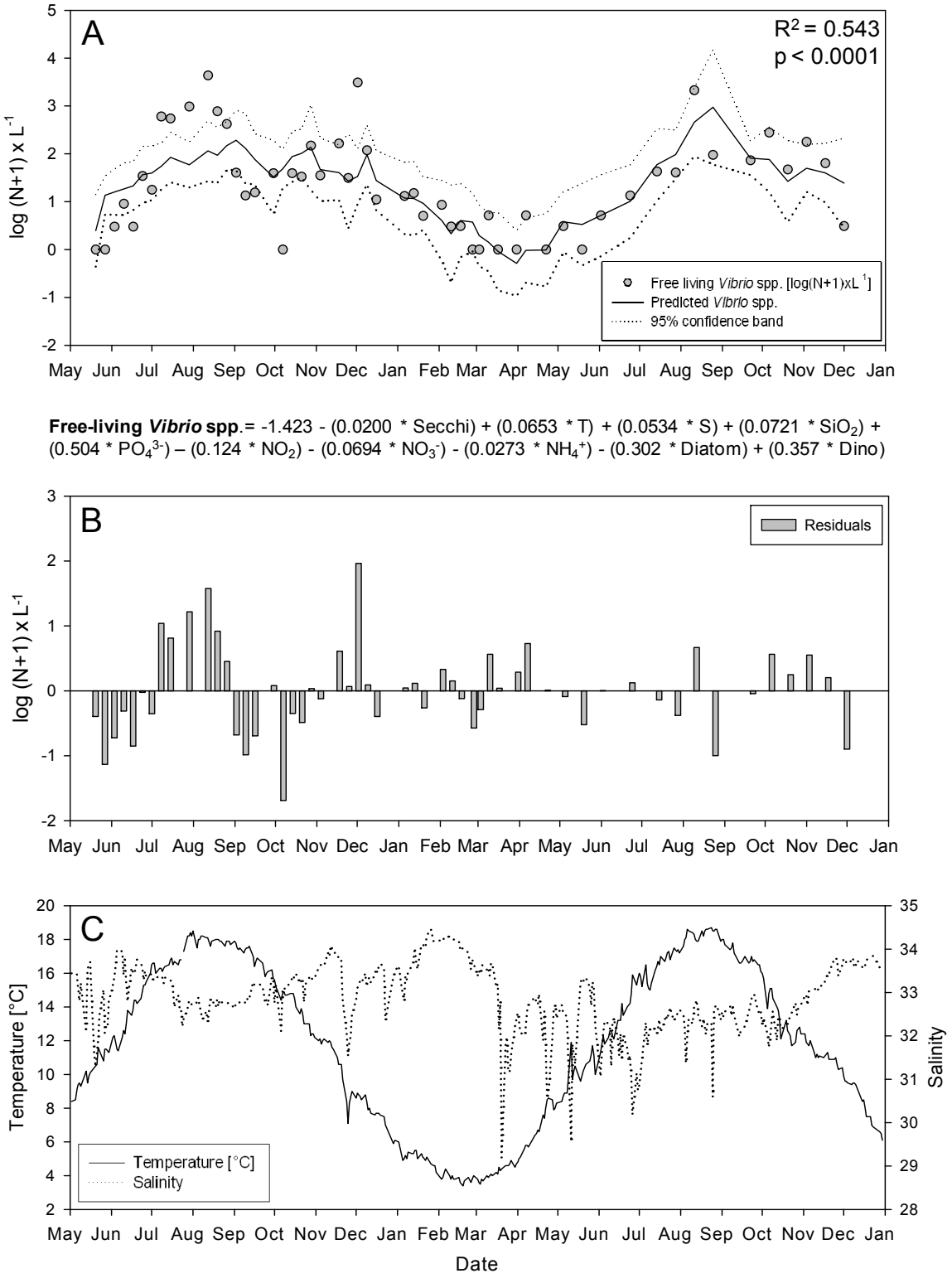

FIGURE 2. Observed and predicted abundances of free-living *Vibrio* spp. between May 2008 and December 2009 (A). Predictions are based on a multiple regression model; corresponding residuals for *Vibrio* spp. abundances are displayed (B). Data for temperature and salinity during the sampling period are given (C).

Based on this tree, the *Vibrio* isolates from Helgoland Roads could be differentiated into six distinct groups (Figure 3A). No clustering or specific allocation of strains in the tree concerning their sampling source could be detected. Three isolates each fell into a *V. harveyi* and a *V. vulnificus* group. A third group consisting of reference sequences of *V. mimicus*, *V. cholerae* and *V. metschnikovii* harbored 10 (6%) isolates from Helgoland Roads. Due to the emphasis of this study on *V. parahaemolyticus* and *V. alginolyticus*, the latter three *Vibrio* groups were omitted from further analyses. The *V. parahaemolyticus* and *V. alginolyticus* strains could be assigned to three phylogenetically distinct groups: The *V. parahaemolyticus* group containing 15 strains (9%), the *V. alginolyticus* group I containing 55 strains (33%) and the *V. alginolyticus* group II containing 80 strains (48%). The *V. parahaemolyticus* and the *V. alginolyticus* group II included reference strains from the respective *Vibrio* species. For example, the *V. parahaemolyticus* branch of the ARB tree is shown in detail in Figure 3B. *V. alginolyticus* group I did not contain any reference strain sequences, but due to the close phylogenetic similarity to *V. alginolyticus* group II, we assumed that the 55 isolates from Helgoland Roads in group I belonged to the species *V. alginolyticus*.

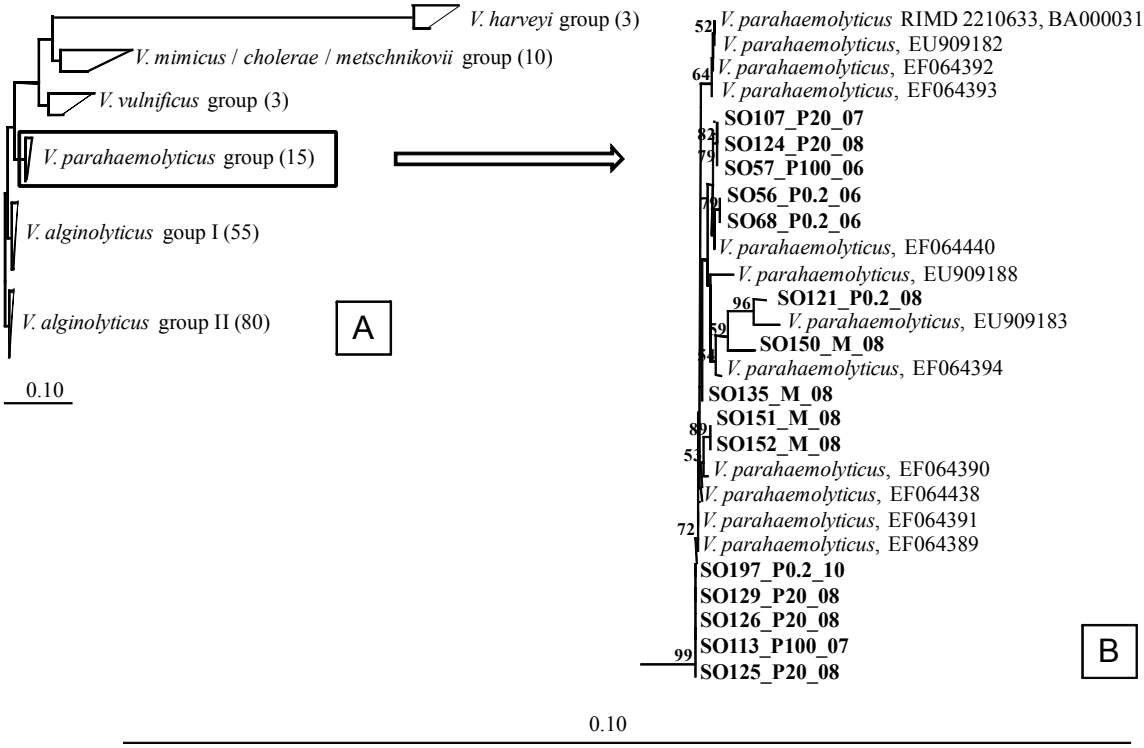

FIGURE 3. Phylogenetic tree of members of the genus *Vibrio* based on *rpoB* sequences (A) and *V. parahaemolyticus* group in detail (B); Scale bars represent 10 nucleotide substitutions per 100 nucleotides. A: Numbers of Helgoland strains in groups are given. B: Strains from Helgoland (SO) isolated from *M. edulis* (M) or different plankton fractions (P0.2, P20, P100) in bold. GenBank accession numbers and bootstrap values >50% are displayed.

Seasonal occurrence of *V. parahaemolyticus*, *V. alginolyticus* I, *V. alginolyticus* II

The occurrence of the three main groups from the *rpoB* ARB tree between April and December 2009 is, subdivided into the fractions '> 0.2 µm', '> 20 µm','> 100 µm' and *M. edulis*, illustrated in Figure 4. In July and August at least two *Vibrio* groups were present in all sampling sources concurrently, whereas in April and December no *Vibrio* strains could be cultivated. In the fractions '> 0.2 µm', '> 20 µm','> 100 µm' *V. parahaemolyticus* and *V. alginolyticus* were detected over a longer time period, when compared to *M. edulis*. In general the group *V. alginolyticus* II occurred most consistent throughout the year.

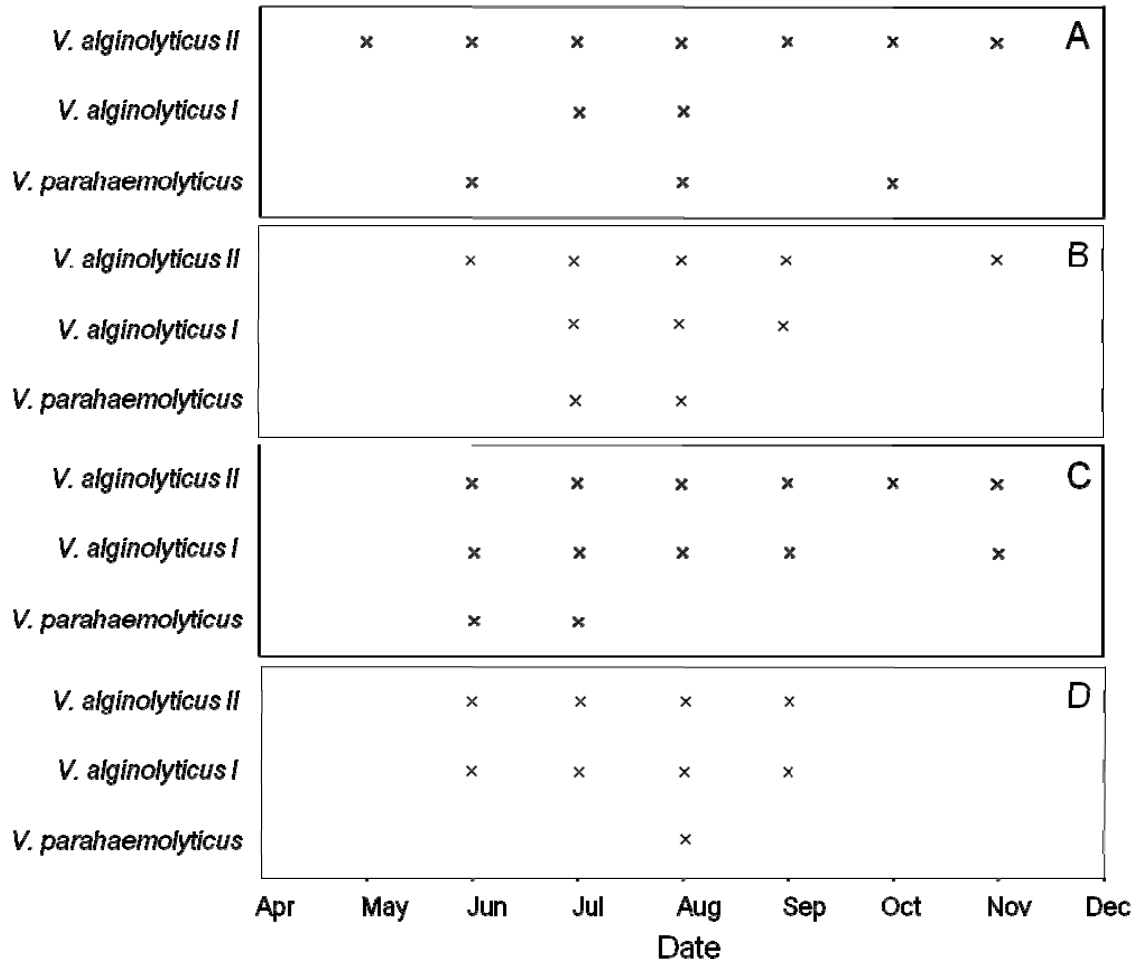

FIGURE 4. Occurrence of groups *V. alginolyticus* I, II and *V. parahaemolyticus* (grouping due to *rpoB* gene sequencing) between April and December 2009. Bacteria cells were free-living (A), >20 µm-attached (B), >100 µm-attached (C) or *M edulis*-associated (D).

Detection of regulatory and virulence-related genes

All 15 isolates from Helgoland Roads assigned to *V. parahaemolyticus* by *rpoB* gene sequencing, were screened for the species-specific gene, *toxR*, to confirm the findings of *rpoB* sequencing. To test further the pathogenicity of the strains, we applied PCR targeting the virulence-associated genes *tdh* and *trh*. All 15 isolates were positive for the gene *toxR*, while the genes *tdh* and *trh* could not be detected in any of the *V. parahaemolyticus* isolates.

Genomic fingerprinting (ERIC-PCR) and multivariate statistical analysis

To investigate the intraspecies diversity of the *V. parahaemolyticus* and *V. alginolyticus* populations, genomic fingerprints of 147 isolates from Helgoland Roads assigned to the *rpoB* groups *V. parahaemolyticus* and *V. alginolyticus* I / II were created using ERIC-PCR. From fingerprints of all examined isolates, 41 different band classes were extracted and a band matching table was created. Based on a similarity matrix (Jaccard index) of the ERIC-PCR band classes, ANOSIM was performed. The ANOSIM calculation proved the congruence of the *rpoB* gene classification and the ERIC fingerprinting (global R = 0.689). All three *rpoB* groups showed highly significant separation based on the fingerprint pattern (level 0.1). ANOSIM pairwise testing revealed R-values of 0.922 (*alginolyticus* I, *parahaemolyticus*), 0.759 (*alginolyticus* II, *parahaemolyticus*) and 0.643 (*alginolyticus* I, *alginolyticus* II). The ordination of the *V. parahaemolyticus* and *V. alginolyticus* strains based on the ERIC fingerprints and their classification due to *rpoB* gene sequencing is represented in an MDS plot (Figure 5). Also included is the information regarding the sampling month of the strains. Most strains from *rpoB* group *V. alginolyticus* II were sampled in late summer to autumn. Whereas strains sampled in August dominate the *rpoB* group *V. parahaemolyticus*.

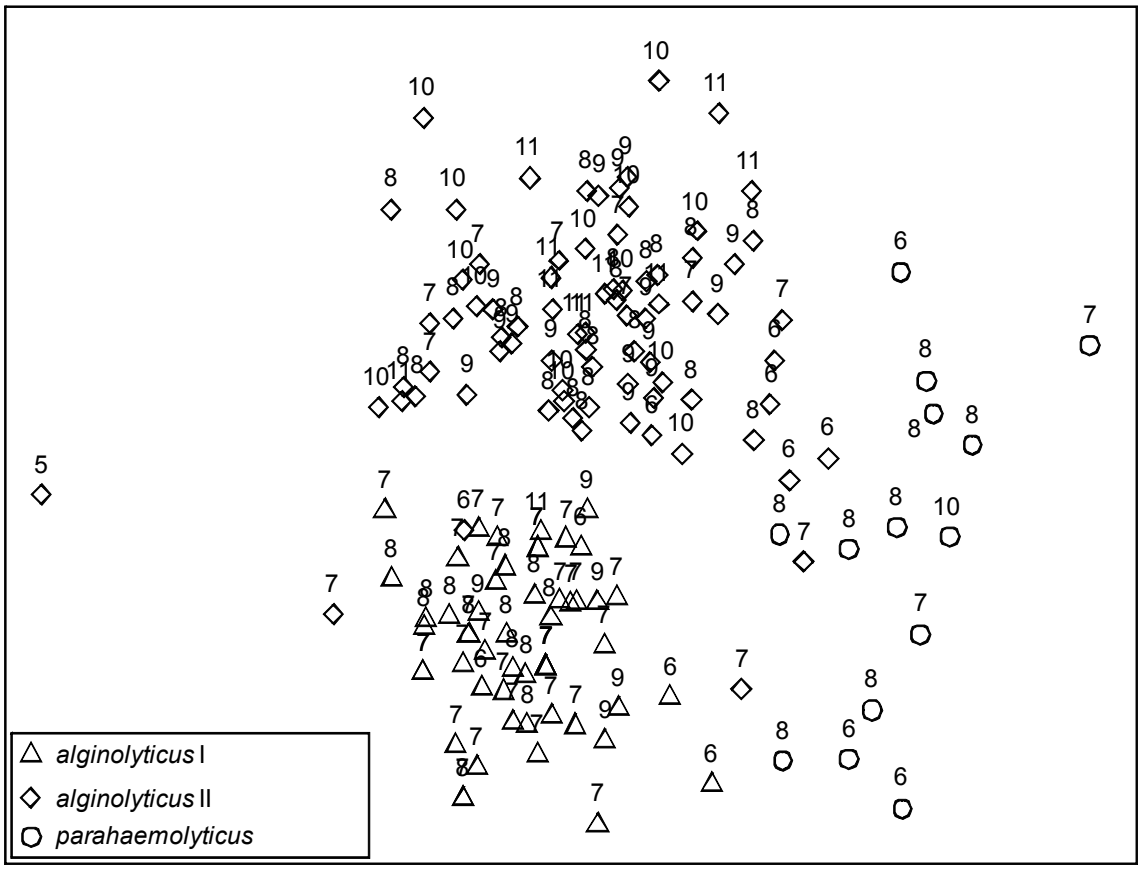

FIGURE 5. MDS plot for Jaccard similarities of ERIC fingerprints including information about *rpoB* grouping. Labels according to sampling month (year 2009). Two-dimensional stress value is 0.23.

To estimate the response to environmental parameters within the single groups *V. parahaemolyticus*, *V. alginolyticus* I and *V. alginolyticus* II, we performed correspondence analyses (CAs) as biplots individually for the *rpoB* subgroups based on the ERIC fingerprints. The results of the CAs illustrated that the environmental parameters had a distinct influence on the structure of the *Vibrio* groups. As an example the CA of the group *V. parahaemolyticus* is shown in Figure 6. The length of the arrows displays the extent of the influence of the corresponding parameter. In case of the *V. parahaemolyticus* group, temperature, salinity and NO_3^- appear to have the greatest influence. Dinoflagellates, SiO_2, secchi depth and NO_2 were shown to have a strong influence on the structure of the *V. parahaemolyticus* group, as well. The ordination within the group *V. alginolyticus* I was mainly influenced by the secchi depth, NH_4^+ concentrations and diatoms, whereas the *V. alginolyticus* II group was more influenced by PO_4^{3-} and NH_4^+ (data not shown).

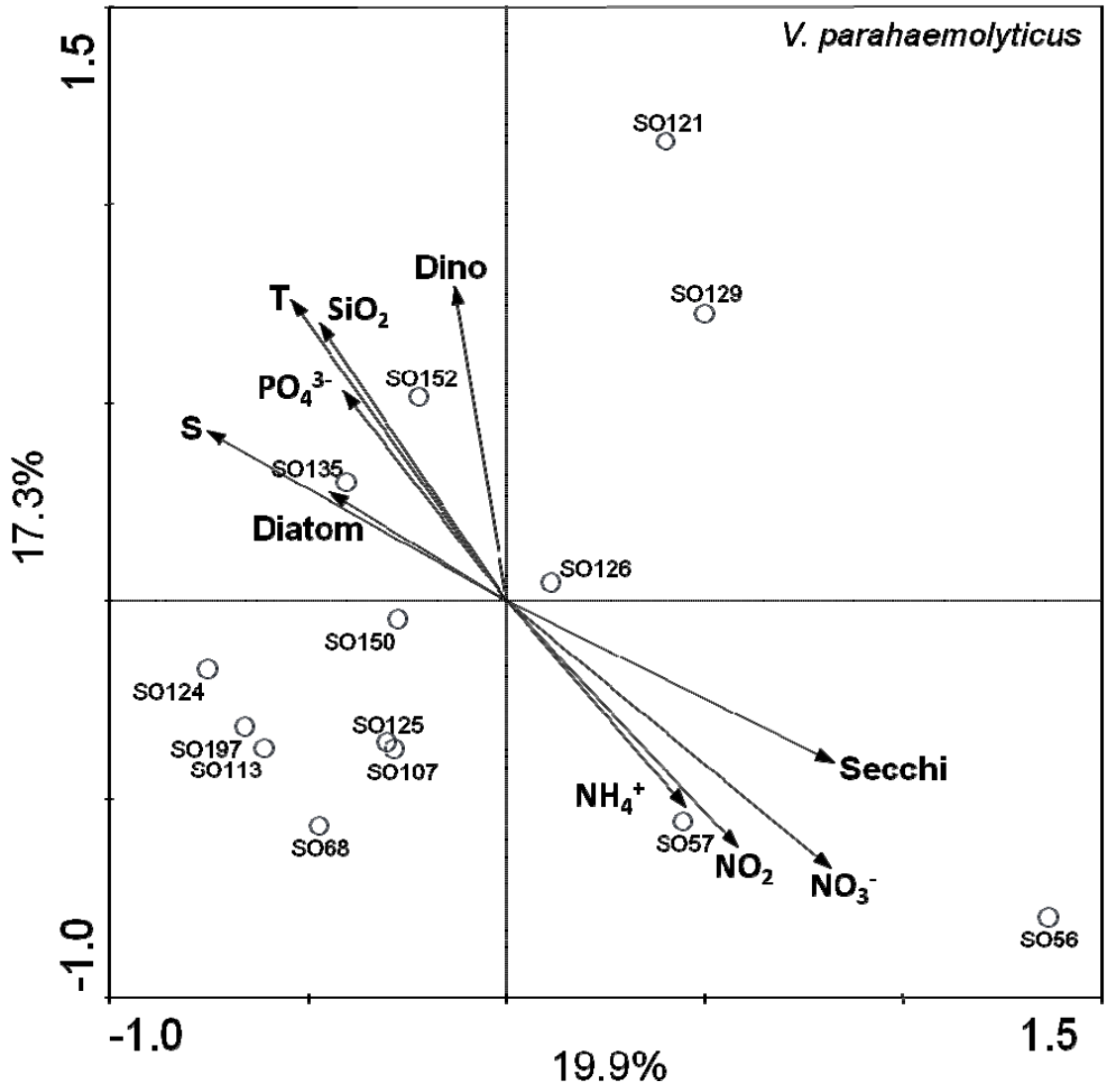

FIGURE 6. Biplot of correspondence analysis (CA) displaying environmental parameters and population structure of *V. parahaemolyticus* based on ERIC fingerprints. Length of arrows indicates extent of influence of parameters on population structure.

Discussion

In this study we investigated the composition, dynamics and response of the *Vibrio* spp. community at Helgoland Roads (North Sea, Germany) to environmental parameters. Special emphasis was placed on the *V. parahaemolyticus* and *V. alginolyticus* populations. In detail, we investigated the following questions: (1) How abundant are mesophilic *Vibrio* spp. at Helgoland Roads? (2) What *V. parahaemolyticus* / *V. alginolyticus* populations do we find at Helgoland Roads? (3) Which environmental parameters affect this population through the year? (4) Can we use these observations to make predictions concerning the pathogenic potential of *Vibrio* communities in temperate waters?

The abundances of free-living, plankton-attached and shellfish-associated *Vibrio* spp. at Helgoland Roads were determined. To target solely potentially human pathogenic strains, a cultivation approach was performed using selective conditions with respect to medium (TCBS) and temperature (37° C). With peaks of 2-4 x 10^3 free-living cells x L^{-1}, the *Vibrio* abundances ranged slightly below comparable studies from the USA and Europe (Hsieh*, et al.*, 2007, Gugliandolo*, et al.*, 2008). This may be due to the fact that the sampling station, Helgoland Roads, experiences comparatively lower water temperatures. Data from sampling stations with the same conditions are hardly available, as investigations of mesophilic *Vibrio* spp. in temperate, Northern European waters are rare (Bauer*, et al.*, 2006, Baker-Austin*, et al.*, 2010). Virtually no *Vibrio* spp. could be cultivated at Helgoland Roads below seawater temperatures of 8°C. That is due to the fact that *Vibrio* bacteria enter a viable but non-culturable (VBNC) state at unfavorable environmental conditions, such as cold water temperatures (Ravel*, et al.*, 1995, Baffone*, et al.*, 2003, Oliver, 2005). At water temperatures above 8°C, free-living *Vibrio* spp. displayed three abundance peaks between May 2008 and January 2010 (Figure 2A). From March 2009, also the abundances of plankton-attached *Vibrio* spp. were detected ('> 20 µm', '> 100 µm'). We aimed to assess a seasonal trend of *Vibrio* abundances comparing different water fractions with each other. This is why, in contrast to other studies (Rawlings*, et al.*, 2007, Turner*, et al.*, 2009), the data for attached *Vibrio* spp. in this study were indicated per volume seawater instead of per weight / surface plankton. Our results revealed that the plankton-attached *Vibrio* spp. at Helgoland Roads follow the same trend as the free-living *Vibrio* spp. (Figure 1). The

abundance peak of *Vibrio* spp. in *M. edulis* occurred one month earlier than the *Vibrio* spp. abundance peak in the fractions '> 0.2 µm', '> 20 µm' and '> 100 µm' (Figure 1). This early peak may be an accumulation effect due to the filter feeding lifestyle of *M. edulis*. Once the *Vibrio* concentration in the ambient water has reached its seasonal peak, a slowed filtration rate or antibacterial activity may have reduced the *Vibrio* abundances in the shellfish tissues already (Hubert, *et al*., 1996, Defer, *et al*., 2009, Terzi & Gucukoglu, 2010). The *Vibrio* spp. in *M. edulis* tissue from Helgoland Roads reached MPN values of 0-240 MPN x g^{-1}. Compared to the MPN values from Helgoland Roads, the quantified *Vibrio* spp. in *M. edulis* from the Oosterschelde (North Sea, the Netherlands) were equal or even lower (6-62 MPN x g^{-1}) (Schets, *et al*., 2010). Though there are no threshold data for total *Vibrio* spp., critical numbers for *V. parahaemolyticus* in food have been defined. According to the U.S. Food and Drug Administration (USFDA), $\geq 1 \times 10^4$ *V. parahaemolyticus* cells per gram seafood represent a potential threat to human health (USFDA, 2001a). The UK Health Protection Agency defines the infection hazard as moderate, when $20 \geq 1 \times 10^3$ CFU *V. parahaemolyticus* per gram ready-to-eat food are detected, and as high, when $> 1 \times 10^3$ CFU are detected (Health Protection Agency, 2009). In Germany, no food or bathwater inspection concerning *Vibrio* spp. is mandatory so far. The *Vibrio* counts in shellfish and water from Helgoland are not alarming, but should be observed over a longer time scale.

Since the second aim of this study was to more closely examine the *V. parahaemolyticus* / *V. alginolyticus* populations of Helgoland Roads, we cultivated *Vibrio* strains and classified them phylogenetically, according to a previously performed method evaluation (Oberbeckmann*, et al.*, 2011). About 80% of the isolates were classified as *V. alginolyticus*, which is in agreement with other studies from European waters (Cavallo & Stabili, 2002, Lhafi & Kühne, 2007). The second most abundant culturable *Vibrio* species was the potential human pathogen, *V. parahaemolyticus*, an observation likewise supported by previous studies (Lhafi & Kühne, 2007, Deter, *et al.*, 2010a). Only few of our isolated strains (<10%) belonged to other *Vibrio* species. Upon close examination of the phylogenetic *rpoB* tree, it is notable that *V. alginolyticus* could be divided into two groups: One group containing *V. alginolyticus* reference strains from the GenBank database (group II), and another one with no reference strains (group I). However, group I showed a very close phylogenetic similarity to group II, which led

us to assume that group I belonged to the species *V. alginolyticus*. Nonetheless, *V. alginolyticus* group I and II were genetically distinguishable and group I may represent a transition group between *V. parahaemolyticus* and *V. alginolyticus*. To the best of our knowledge, no such transition group has been reported in previous studies. Solely hybrid strains within other *Vibrio* species, such as *V. cholera* or *V. vulnificus* have been described elsewhere (Bisharat, *et al*., 2007, Choi, *et al*., 2010b). However, the separation between the *V. alginolyticus* groups I and II from Helgoland Roads and between these two groups and the *V. parahaemolyticus* group was supported by the results of the ERIC-PCR fingerprinting. The ANOSIM calculations based on the fingerprint results proved a significant separation between the *rpoB* groups, which is illustrated in Figure 5. The timescale (Figure 4) shows that all three *Vibrio* groups (*V. parahaemolyticus*, *V. alginolyticus* I and II) coexisted solely in the summer months, indicating a higher diversity concerning *Vibrio* spp.. A study by Deter and coworkers (2010a) in northern France corroborates this observation. The authors detected the highest biodiversity within the *Vibrio* community in late summer. When comparing the occurrence of the species *V. alginolyticus* and *V. parahaemolyticus*, our results show that *V. alginolyticus* has a broader occurrence range throughout the year (Figures 4 and 5). The *V. alginolyticus* group II was cultivable from May to November, the probable transition group *V. alginolyticus* group I mostly from June to September. The potentially pathogenic species *V. parahaemolyticus* was only cultivable in certain months with high water temperature, especially in August.

Several environmental parameters, such as temperature, salinity or plankton occurrence, have been reported to affect *Vibrio* communities' worldwide (Thompson, *et al*., 2004b, Drake, *et al*., 2007, Blackwell & Oliver, 2008, Turner, *et al*., 2009, Vezzulli, *et al*., 2009). But profound studies on the responses of *Vibrio* spp. to environmental parameters in Northern European waters are nearly missing. One aim of this study was to assess these responses at Helgoland Roads (North Sea, Germany). Our results revealed that free-living, plankton-attached and shellfish-associated *Vibrio* spp. were mainly influenced by the same environmental parameters (Table 3). To go more into detail, free-living *Vibrio* spp. displayed three peaks between May 2008 and January 2010 (Figure 2A), two of which occurred in August (2008 and 2009), when highest temperatures (>18°C) and comparably high phytoplankton densities were measured. Several previous studies attributed sea water temperature to be a key factor for the

occurrence of *Vibrio* spp. (Sobrinho*, et al.*, 2010, Vezzulli*, et al.*, 2010). Also parameters such as salinity, phytoplankton and nutrients are known to have an effect on *Vibrio* abundances as well (Martinez-Urtaza, *et al*., 2008b, Turner, *et al*., 2009, Vezzulli, *et al*., 2009, Caburlotto, *et al*., 2010b). This is supported by the third abundance peak of free-living *Vibrio* spp. at Helgoland Roads, detected in December 2008. This peak was recorded concurrent with a remarkable drop of salinity and temperature and a slight increase of nutrients and dinoflagellates. The sudden change of environmental parameters implies a shift of coastal waters towards Helgoland Roads. Even though the temperature was lower, the *Vibrio* spp. abundance in the coastal water body was higher than in the more marine water body at Helgoland Roads. Hence, other parameters such as low salinity and increased phytoplankton and nutrients also appear to affect *Vibrio* spp. abundances at Helgoland Roads. In this study, the Spearman correlation analysis revealed that the *Vibrio* abundances at Helgoland Roads were significantly correlated with temperature and nutrient concentrations (Table 3). The algal groups did not show significant correlations with *Vibrio* spp. isolated from any water or shellfish fraction, but it is known that algae influence *Vibrio* communities (Mourino-Perez*, et al.*, 2003, Eiler*, et al.*, 2006, Turner*, et al.*, 2009). Most likely, the effect of algae on *Vibrio* abundances at Helgoland Roads was not covered by the measured parameter. However, CA biplots showed that dinoflagellates and diatoms had an influence on the structure of the *V. parahaemolyticus* and *V. alginolyticus* populations at Helgoland Roads (e.g. Figure 6).

The salinity did not show a significant Spearman correlation with the *Vibrio* abundances, although the highest *Vibrio* abundances occurred when salinity was reduced (Figure 2). This might be due to the fact that salinity did not correlate with *Vibrio* abundances constantly over the year, but rather seemed to influence the *Vibrio* abundances only at certain events (shift of coastal water). Also the multiple regression model and the corresponding residual analysis implied that certain events of low salinity influence *Vibrio* spp. at Helgoland Roads. The observed abundances of free-living *Vibrio* spp. agreed significantly with the predicted abundances, but during some events with reduced salinity, the detected *Vibrio* abundances were clearly higher than the predicted ones (Figure 2). Previous studies postulated that similarly high temperatures combined with events of low salinity promote *Vibrio* spp. and especially the species *V. parahaemolyticus* (Martinez-Urtaza, *et al*., 2008b). Our results indicate that periods of high temperature and lower salinity should be investigated further to make robust

models predicting the abundance of potentially pathogenic *Vibrio* spp. in temperate waters, such as the German Bight.

To more closely examine which parameters have an influence on the community structure within individual *Vibrio* populations, correspondence analysis (CA) was performed on the three subgroups *V. parahaemolyticus* and *V. alginolyticus* I / II. These analyses revealed that the ERIC band pattern of the isolates did not occur randomly, but that the population patterns were distinctly influenced by environmental parameters. Each *Vibrio* group was influenced by a different combination of environmental parameters. No single environmental parameter could explain the whole structure of the *V. alginolyticus* and *V. parahaemolyticus* populations, but rather a combination of parameters was needed. This is supported by other recent studies in this field (Hsieh, *et al*., 2008, Turner, *et al*., 2009, Deter, *et al*., 2010b). As an example, the parameters most strongly influencing the *V. parahaemolyticus* population were temperature, salinity and NO_3^- (Figure 6). Also the Spearman correlation analysis revealed that temperature and NO_3^- correlated significantly with the abundances of free-living, plankton-attached and shellfish-associated *Vibrio* spp. (Table 3). Water temperature correlated positively, NO_3^- negatively with *Vibrio* numbers. Most likely, the negative correlation of NO_3^- and *Vibrio* spp. does not display a direct effect. Low NO_3^- concentration rather reflects a hydrographical regime including high phytoplankton abundances, which is favorable for *Vibrio* spp..

Based on this study we can state that the *V. parahaemolyticus* population at Helgoland Roads is structured by different environmental parameters, especially temperature. Our results indicate that summer is the season with the highest risk for infections caused by *V. parahaemolyticus*. Incorporating the influence of environmental parameters on the pathogenicity of *V. parahaemolyticus*, Rodriguez-Castro and coworkers (2010) identified an influence of the water temperature on the presence of the genes *tdh* and *trh*, and Whitaker and coworkers (2010) showed that low salinity increases the toxicity of *V. parahaemolyticus* strains. Another study revealed that the expression of certain virulence-associated traits, such as hemolysin, cytotoxicity, biofilm formation or motility, is correlated with increased temperature (Mahoney*, et al.*, 2010). These observations are alarming in the light of climate change (De Toni*, et al.*, 2009, Coppola & Giorgi, 2010, Mills*, et al.*, 2010). An alteration of environmental parameters, e.g. rising water temperatures, might lead to a *Vibrio* community shift towards potentially

pathogenic species and strains. For the German Bight (North Sea), a warming trend has been reported and other environmental parameters are changing constantly in this and other temperate European waters (Wiltshire & Manly, 2004, Dulvy, *et al.*, 2008, Belkin, 2009, Wiltshire *et al.*, 2010). Thus far, we did not detect the virulence-associated genes *tdh* or *trh* in the strains from Helgoland Roads. However, this is not surprising considering that only a minor percentage of environmental strains contain the genes *tdh* or *trh* (Martinez-Urtaza, *et al*., 2008b, Rodriguez-Castro, *et al*., 2010). Furthermore, several other genes are involved in the pathogenicity process of *V. parahaemolyticus* (Caburlotto, *et al*., 2009, Caburlotto, *et al*., 2010c) and the difference between pathogenic and non-pathogenic strains is not a matter of a single gene (Izutsu*, et al.*, 2008). The environment, both sediment and water bodies, can be seen as reservoir for pathogenicity factors and changing environmental parameters might favor the transmission of these factors. In the North Sea and other dynamic temperate ecosystems with changing environmental conditions, such as increasing water temperatures, further monitoring of *Vibrio* spp. is therefore essential.

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CHAPTER III

Seasonal dynamics and predictive modeling of a *Vibrio* community in coastal waters of the North Sea

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Abstract

Vibrio spp. are ubiquitous members of marine waters all over the world. High genome plasticity due to frequent mutation, recombination and lateral gene transfer, enables *Vibrio* bacteria to adapt rapidly to environmental changes. The genus *Vibrio* harbors several human pathogens, which commonly cause outbreaks in tropical regions. In recent years, pathogenic *Vibrio* strains emerged also in European waters. Little is known about factors driving the spread of *Vibrio* spp. in temperate waters such as the North Sea. This study represents a cultivation-independent approach to quantify *Vibrio* bacteria in the North Sea and assess their response to biotic and abiotic parameters. Between January and December 2009, *Vibrio* abundances at Helgoland Roads (North Sea, Germany) were estimated using fluorescence *in situ* hybridization (FISH). *Vibrio* numbers up to 3.37 x 10^4 cells x mL^{-1} (2.22% of total bacterial counts) were determined. Correlations between *Vibrio* spp. and nutrients (SiO_2, PO_4^{3-}, NO_2, NO_3^-, NH_4^+), secchi depth, temperature, salinity, chlorophyll a and phytoplankton (diatoms and dinoflagellates) were calculated using Spearman Rank analysis. Multiple regression analysis was carried out to analyze the additive influence of multiple factors on *Vibrio* spp., using either all measured parameters (full model) or solely temperature and salinity (T/S model) as independent variables. Based on these calculations we report that high water temperature, low salinity and high algae abundances can favor the increase of *Vibrio* spp.. We state further that *Vibrio* abundances are influenced by a complex combination of environmental parameters and are subject to seasonal dynamics at Helgoland Roads. Ongoing investigations are essential to disclose the dynamics of *Vibrio* spp. in the course of climate change, also in European waters. Multiple regression models could improve and simplify such investigations, since they represent an efficient and reliable tool to estimate *Vibrio* abundances in the water.

Introduction

European temperate waters, such as the North Sea, are exposed to manifold anthropogenic influences. The North Sea is economically important with shipping lanes, harbors, fisheries, industry, tourism, recreation and as rich source of energy resources. Effects of climate change have been documented for the North Sea: Over the last 45 years a significant increase of temperature could be detected (Belkin, 2009, Wiltshire*, et al.*, 2010). Besides anthropogenic influences, natural freshwater inflow, tides, currents and water circulation make the North Sea a highly dynamic ecosystem. Bacterial communities are defined by their environment and respond very rapidly to both anthropogenic and natural dynamics (Miller*, et al.*, 2009, Alonso*, et al.*, 2010, Piquet*, et al.*, 2010). Existing microbial communities have been shown to react immediately to biotic and abiotic factors, such as ambient phytoplankton composition (Jones*, et al.*, 2007, Sapp*, et al.*, 2007, Campbell*, et al.*, 2009). New strains can arise in a system with changing environmental parameters including temperature, and several of recently emerging bacteria strains in European waters have been reported to be human pathogens (Martinez-Urtaza*, et al.*, 2005, Soto, 2009). The effects of the environmental dynamics on bacterial communities of the North Sea and in particular on pathogenic strains are still not well understood (Sapp*, et al.*, 2010).

This study focuses on the genus *Vibrio* in the temperate waters of the North Sea (Helgoland, Germany). Bacteria of the genus *Vibrio* are ubiquitous in sediments and marine and brackish waters worldwide. They are found free-living or associated with zoo- and phytoplankton (Thompson, *et al*., 2004a, Farmer, *et al*., 2005). These bacteria are gram-negative, motile, mostly halophilic and extremely adaptable to their environment. Several *Vibrio* species are known human pathogens, such as *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. These pathogens are transmitted to humans via contaminated water or raw seafood and represent one of the main causes for foodborne diseases worldwide (Daniels & Shafaie, 2000, Su & Liu, 2007, Jones & Oliver, 2009). In recent years pathogenic *Vibrio* spp. strains have been found in temperate European waters (Baker-Austin*, et al.*, 2010). However, investigations of the *Vibrio* communities in these waters are still rare and standardized monitoring is lacking. Little is also known about the seasonal dynamics of *Vibrio* communities in temperate European waters and their response to environmental parameters.

This study aims to close this knowledge gap and represents one of the first cultivation-independent investigations of the *Vibrio* community in the North Sea. The abundance of *Vibrio* spp. was determined using fluorescence *in situ* hybridization (FISH), with the *Vibrio*-specific and frequently applied probe 'GV' (Giuliano*, et al.*, 1999, Eilers*, et al.*, 2000). The corresponding environmental data, namely temperature, salinity, nutrient and chlorophyll a concentrations, algae abundances and secchi depth, originate from a long-term data series, which has been in place for over 40 years at Helgoland Roads (Wiltshire & Manly, 2004). By multiple regression analyses the diverse influences of environmental parameters on the *Vibrio* community were calculated.

Material and Methods

Sample collection

Seawater samples were taken twice a week at Helgoland Roads (North Sea, Germany, 54°11.3 ‘N, 7°54.0 ‘E) from January - November 2009 for FISH analysis. Corresponding analysis of nutrients (SiO_2, PO_4^{3-}, NO_2, NO_3^-, NH_4^+), secchi depth, temperature, salinity, chlorophyll a and phytoplankton abundances (diatoms and dinoflagellates) was carried out (Table 1).

TABLE 1. List of environmental parameters, abbreviations, units and measurement methods / instruments.

Parameter	Abbreviation	Unit	Measurement
Secchi depth	Secchi	m	Secchi disk (directly on board ship)
Temperature	T	°C	Thermometer (directly on board ship)
Salinity	S		Salinometer Autosal, Guideline
SiO_2		µmol x L^{-1}	Chemical / photometric determination (Grasshoff *et al.*, 1999)
PO_4^{3-}		µmol x L^{-1}	
NO_2		µmol x L^{-1}	
NO_3^-		µmol x L^{-1}	
$NH4^+$		µmol x L^{-1}	
Diatoms		N x L^{-1}	Utermöhl counting (Utermöhl, 1958) Inverted microscope Axiovert 135
Dinoflagellates	Dino	N x L^{-1}	
Chlorophyll a	Chla	µg x L^{-1}	BBE Algae Analyser, Moldaenke

Fixation and CARD-FISH

For the quantification of *Bacteria*, *γ-Proteobacteria* and *Vibrio* spp., catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) was performed. Samples for CARD-FISH were fixed with sterile filtered, 37% formaldehyde solution (final concentration 1% v/v) for 2 h at room temperature. Water samples of 10 mL (for DAPI counts of total cells and quantification of *Bacteria* and *γ-Proteobacteria*) and 100 mL (for quantification of *Vibrio*) were filtered onto polycarbonate filters (type GTTP; 0.2 µm pore size; 47 mm diameter), which were frozen at -20°C for further analyses. CARD-FISH was performed according to Pernthaler and coworkers (2004), with the following modifications described by Schattenhofer and coworkers (2009): The hybridization was carried out over night and the tyramide signal amplification was carried out for 45 min. Both steps were performed at 46°C. The filter sections were washed twice in 96% ethanol, dried and embedded in the antifading reagent

VECTASHIELD® on microscope slides. For the quantification of total microbial cell numbers, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI, 1 µg mL^{-1}). Both, hybridized and DAPI-stained cells were quantified on an Axioplan II Imaging epifluorescence microscope (Zeiss).

Hybridization was conducted with the oligonucleotide probes EUB338-I-III (*Bacteria*), GAM42a (γ-*Proteobacteria*), GV (*Vibrio*) and NON (negative control), which were all labeled with horseradish peroxidase (Biomers) (Table 2). The specificity of the probes EUB338-I-III and GAM42a are described in Amann and Fuchs (2008). In the course of this study, the probe specificity of GV was checked against the SILVA 16S rRNA database (Pruesse*, et al.*, 2007).

TABLE 2. List of applied probes including target groups, probe sequences, required formamide concentrations (FA %) and references

Probe	Target group	Probe sequence (5' to 3')	FA (%)	Reference
EUB338-I	*Bacteria*	GCTGCCTCCCGTAGGAGT	35	Amann *et al.* (1990)
EUB338-II		GCAGCCACCCGTAGGTGT	35	Daims *et al.* (1999)
EUB338-III		GCTGCCACCCGTAGGTGT	35	Daims *et al.* (1999)
GAM42a	γ-*Proteobacteria*	GCCTTCCCACATCGTTT	35	Manz *et al.* (1992)
GV841	*Vibrio*	AGGCCACAACCTCCAAGTAG	30	Giuliano *et al.* (1999)

Statistical analysis

To investigate the relationship between the parameters secchi depth, water temperature, salinity, SiO_2, PO_4^{3-}, NO_2, NO_3^-, NH_4^+, chlorophyll a, diatoms, dinoflagellates and the abundances of *Vibrio* spp., statistical analyses were carried out using the software STATISTICA (StatSoft, version 7.1). Correlations between log-transformed *Vibrio* abundances and environmental parameters, as well as between the parameters, were determined using Spearman rank analyses. Missing values were excluded pairwise and the significance level for correlating variables was set to $p < 0.05$.

Multiple regressions were calculated for *Vibrio* abundances (dependent variable), either with all measured environmental parameters as independent variables (full model) or solely with temperature and salinity (T/S model). Important values to describe the results of the multiple regression analysis are R^2, p, Beta, partial and semi-partial correlation and tolerance. Beta is a standardized regression coefficient, which indicates the relative contribution of a single independent variable to the prediction of the dependent variable. Partial correlation is the correlation between the dependent and one

independent variable, after the subtraction of the linear effects of all other independent variables within the regression model. Semi-partial correlation is the correlation between the dependent and one independent variable, after the subtraction of the linear effects of all other independent variables on the corresponding independent variable. The tolerance signifies the extent of the correlation between one independent variable and all other independent variables. A low tolerance value indicates a high autocorrelation between one considered independent variable and the remaining independent variables.

To simplify the full model, only the variables with significant influence ($p < 0.05$) were chosen for the graphic illustration using the software SigmaPlot (Version 11). Not only the models itself, but also the residuals of each model are presented to point out the difference between observed and predicted abundances in detail. Besides using the log-transformed *Vibrio* abundances, all statistical calculations were additionally performed using the percentages of *Vibrio* spp..

Results

CARD-FISH

Before carrying out CARD-FISH, the probe GV was checked for its specificity against the SILVA 16S rRNA database (Pruesse*, et al.*, 2007). The probe validation revealed a coverage of 72% concerning the family *Vibrionaceae*, and a coverage of 88% concerning the genus *Vibrio*. The main species in the genus *Vibrio*, which were not covered by the GV probe, were *V. cholera* and *V. mimicus*. A table of matches and mismatches is available as supplemental material.

The percentages of *Bacteria*, γ-*Proteobacteria* and *Vibrio* spp. of total cells (DAPI counts) between January and December 2009 assessed using CARD-FISH, are presented in Figure 1. *Bacteria*, as detected by the probes EUB338-I-III, represented on most sampling dates between February and April 2009 over 90% of the whole microbial community, with a peak of 96% in mid-April. The percentages of the γ-*Proteobacteria* (detected by the probe GAM42a) reached 2-37% over the year, with two peaks over 35% in March and July. In June and July constantly high percentages of γ-*Proteobacteria* were observed. *Vibrio* percentages, as detected by the probe GV, were highest between May and July. The peak of abundances as well as of percentages of *Vibrio* bacteria was determined in late June with 3.37 x 10^4 cells x mL^{-1} (2.22%). Unlike *Vibrio* spp., the total microbial numbers reached their peak in late August with 3.99 x 10^6 cells x mL^{-1}. The lowest *Vibrio* spp. abundance was 4.97 x 10^2 cells x mL^{-1} in mid-January and in early February. In mid-January the *Vibrio* bacteria represented 0.22% of the whole microbial community. In early February they made up only 0.13%, which was the lowest *Vibrio* percentage throughout the year.

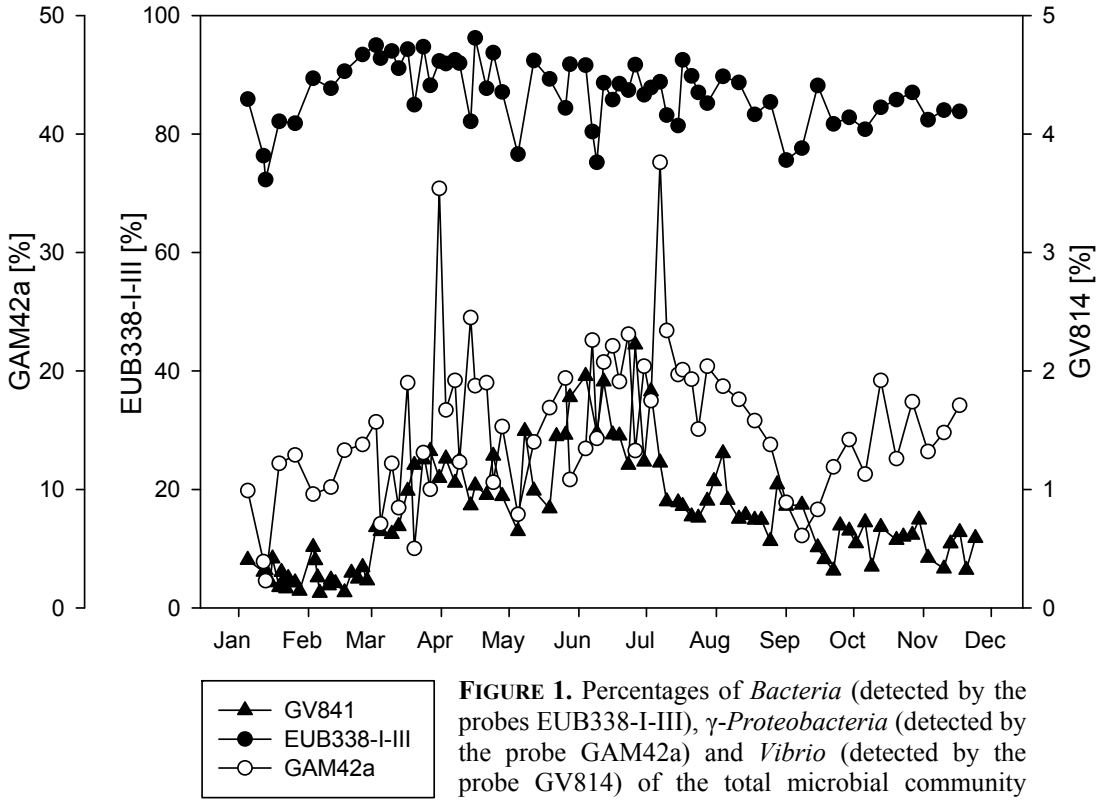


FIGURE 1. Percentages of *Bacteria* (detected by the probes EUB338-I-III), γ-*Proteobacteria* (detected by the probe GAM42a) and *Vibrio* (detected by the probe GV814) of the total microbial community between January and December 2009.

Spearman Correlation Analyses

The trends of the environmental parameters at Helgoland Roads are illustrated in Figure 2. Temperature, algal abundances and chlorophyll a concentrations were lowest between January and March. Temperature reached its peak in August, whereas algal abundances and chlorophyll a showed several peaks between March and September. Salinity was highest between December and February. Several drops in salinity were seen in March, May, June and August. For the nutrients a variety of concentration maxima were detected at Helgoland Roads. SiO_2 peaked in January, March, May and between August and October; PO_4^{3-} in March and August to November and in December; Dissolved inorganic nitrogen (NO_2, NO_3^-, NH_4^+) was highest between March and May and again in December.

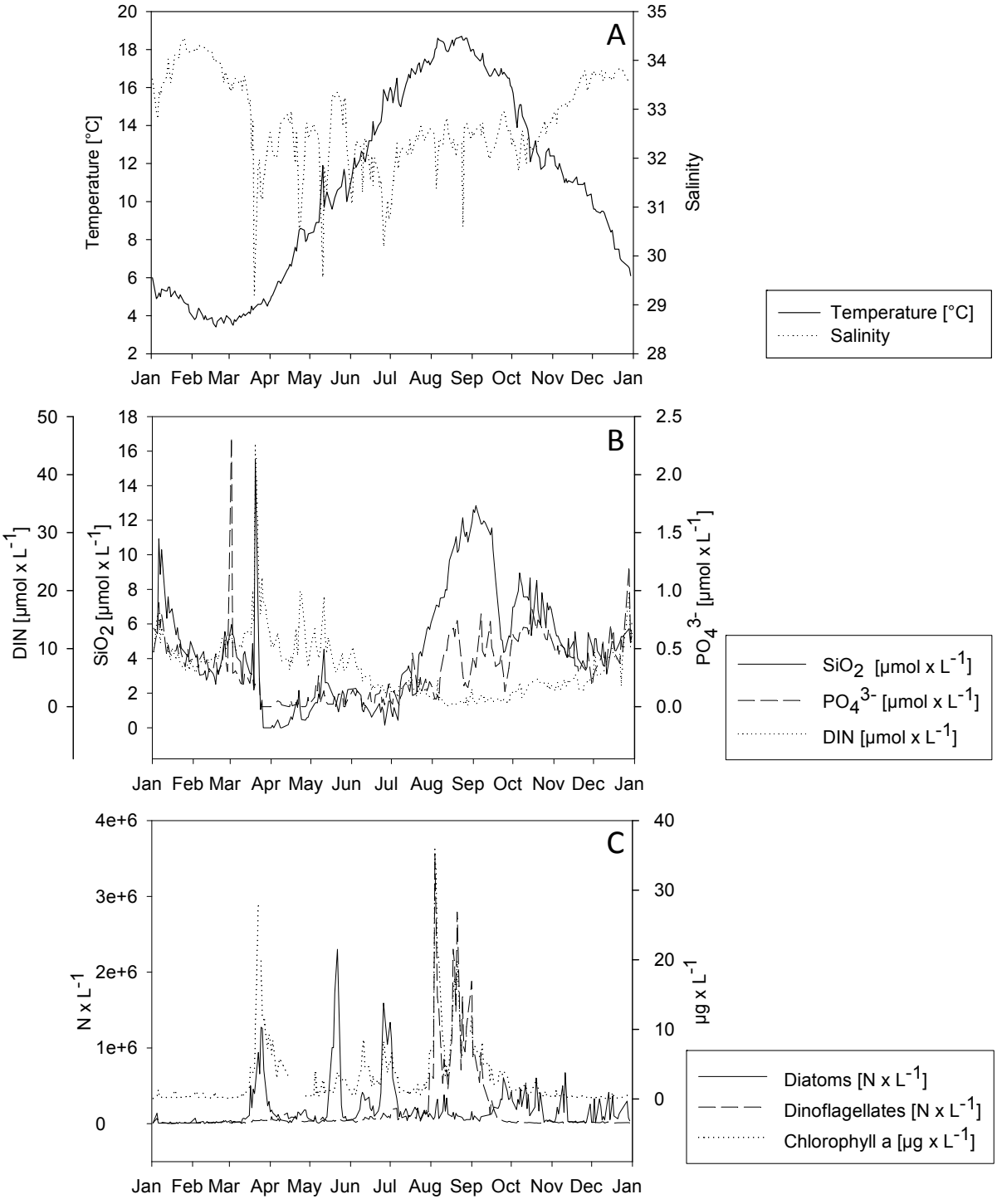


FIGURE 2. Data for water temperature and salinity (A), nutrients (B) and phytoplankton abundances and chlorophyll a concentration (C) between January and December 2009.

These environmental parameters were tested for correlations with log-transformed *Vibrio* abundances as well as with *Vibrio* percentages of total bacteria at Helgoland Roads. The results of these Spearman Rank analyses are listed in Table 3. Except for secchi depth, SiO_2 and NO_3^-, all parameters correlated significantly with both *Vibrio* abundances and percentages. Especially high R-values were obtained for the correlations between *Vibrio* spp. and salinity, dinoflagellates, chlorophyll a and temperature.

TABLE 3. Spearman Rank correlations between environmental parameters and log transformed *Vibrio* abundances (*Vibrio*) or *Vibrio* percentages (%). Significant correlations are given in bold ($p<0.05$).

		Vibrio		%	
Variable	**N**	**R**	**p**	**R**	**p**
Secchi	91	-0.160	0.131	-0.008	0.939
T	91	**0.754**	**<0.001**	**0.454**	**<0.001**
S	91	**-0.790**	**<0.001**	**-0.744**	**<0.001**
SiO_2	91	-0.086	0.420	**-0.400**	**<0.001**
PO_4^{3-}	91	**-0.421**	**<0.001**	**-0.671**	**<0.001**
NO_2	91	**-0.722**	**<0.001**	**-0.419**	**<0.001**
NO_3^-	91	**-0.561**	**<0.001**	-0.181	0.087
$NH4^+$	91	**0.243**	**0.020**	**0.212**	**0.043**
Diatoms	91	**0.647**	**<0.001**	**0.621**	**<0.001**
Dino	91	**0.772**	**<0.001**	**0.637**	**<0.001**
Chla	91	**0.729**	**<0.001**	**0.698**	**<0.001**

We carried out Spearman Rank analyses for the environmental parameters themselves to check autocorrelations between the parameters (Table 4). Over half of the parameters correlated with each other. Highly significant autocorrelation could be detected between temperature and NO_2 / NO_3^-, with negative R-values up to - 0.904.

TABLE 4. Spearman Rank correlations between environmental parameters. Significant correlations are given in bold ($p<0.05$).

	Secchi	**T**	**S**	**SiO_2**	**PO_4^{3-}**	**NO_2**	**NO_3^-**	**$NH4^+$**	**Diatoms**	**Dino**
T	**-0.347**									
S	**0.217**	**-0.656**								
SiO_2	**-0.490**	**0.313**	0.075							
PO_4^{3-}	**-0.359**	-0.018	**0.342**	**0.735**						
NO_2	**0.282**	**-0.904**	**0.578**	**-0.273**	0.011					
NO_3^-	**0.232**	**-0.867**	**0.377**	**-0.370**	-0.128	**0.890**				
$NH4^+$	**-0.235**	0.154	**-0.235**	-0.205	-0.091	-0.114	-0.101			
Diatoms	-0.172	**0.545**	**-0.705**	-0.102	**-0.242**	**-0.559**	**-0.413**	0.205		
Dino	-0.080	**0.690**	**-0.667**	-0.019	**-0.346**	**-0.650**	**-0.517**	0.089	**0.558**	
Chla	-0.117	**0.611**	**-0.683**	-0.086	**-0.368**	**-0.608**	**-0.452**	-0.043	**0.710**	**0.773**

Multiple Regression Analyses (whole year)

For *Vibrio* abundances as well as for percentages, multiple regressions were calculated with all measured environmental parameters as independent variables (full models). The parameters secchi depth, salinity, PO_4^{3-}, NO_2, diatom and dinoflagellate significantly influenced the multiple regression model of *Vibrio* abundances (Table 5). Salinity showed the highest Beta value (- 0.278) and NO_2 the highest partial and semi-partial correlation (- 0.400 and - 0.149). The multiple regression model for *Vibrio* percentages was significantly influenced by the variables secchi depth, salinity, SiO_2 and PO_4^{3-}, with the highest Beta and correlation values for SiO_2 and PO_4^{3-} (Beta = - 0.355 and - 0.314). In both full model calculations (for *Vibrio* abundances as well as for percentages) the parameter temperature showed by far the lowest tolerance value (0.076).

TABLE 5. Results of multiple regression analyses with all measured environmental parameters as independent variables. R- and p-values for full models of *Vibrio* abundances and percentages are displayed. Also given are values for Beta, partial/semipartial (PK/SK) correlation, tolerance and R and p for individual variables. Significantly influencing parameters are indicated in bold ($p<0.05$).

	R^2 (model)	p (model)	Parameter	Beta	PK	SK	Tolerance	R^2 (var)	p (var)
Vibrio	**0.897**	**0.000**	**Secchi**	**-0.099**	**-0.236**	**-0.078**	**0.624**	**0.376**	**0.034**
			T	0.129	0.110	0.036	0.076	0.924	0.329
			S	**-0.278**	**-0.364**	**-0.126**	**0.205**	**0.795**	**0.001**
			SiO_2	-0.024	-0.032	-0.010	0.190	0.810	0.777
			PO_4^{3-}	**-0.222**	**-0.345**	**-0.118**	**0.283**	**0.717**	**0.002**
			NO_2	**-0.266**	**-0.400**	**-0.140**	**0.279**	**0.721**	**<0.001**
			NO_3^-	-0.028	-0.035	-0.011	0.155	0.845	0.760
			$NH4^+$	0.058	0.154	0.050	0.743	0.257	0.171
			Diatoms	**0.130**	**0.232**	**0.077**	**0.350**	**0.650**	**0.037**
			Dino	**0.207**	**0.311**	**0.105**	**0.258**	**0.742**	**0.005**
			Chla	-0.037	-0.076	-0.025	0.446	0.554	0.500
%	**0.747**	**0.000**	**Secchi**	**-0.148**	**-0.226**	**-0.117**	**0.624**	**0.376**	**0.043**
			T	0.129	0.070	0.035	0.076	0.924	0.533
			S	**-0.305**	**-0.264**	**-0.138**	**0.205**	**0.795**	**0.017**
			SiO_2	**-0.355**	**-0.294**	**-0.155**	**0.190**	**0.810**	**0.008**
			PO_4^{3-}	**-0.314**	**-0.315**	**-0.167**	**0.283**	**0.717**	**0.004**
			NO_2	-0.108	-0.113	-0.057	0.279	0.721	0.315
			NO_3^-	0.140	0.109	0.055	0.155	0.845	0.334
			$NH4^+$	-0.072	-0.122	-0.062	0.743	0.257	0.277
			Diatoms	0.087	0.101	0.051	0.350	0.650	0.369
			Dino	0.087	0.087	0.044	0.258	0.742	0.440
			Chla	0.059	0.078	0.039	0.446	0.554	0.489

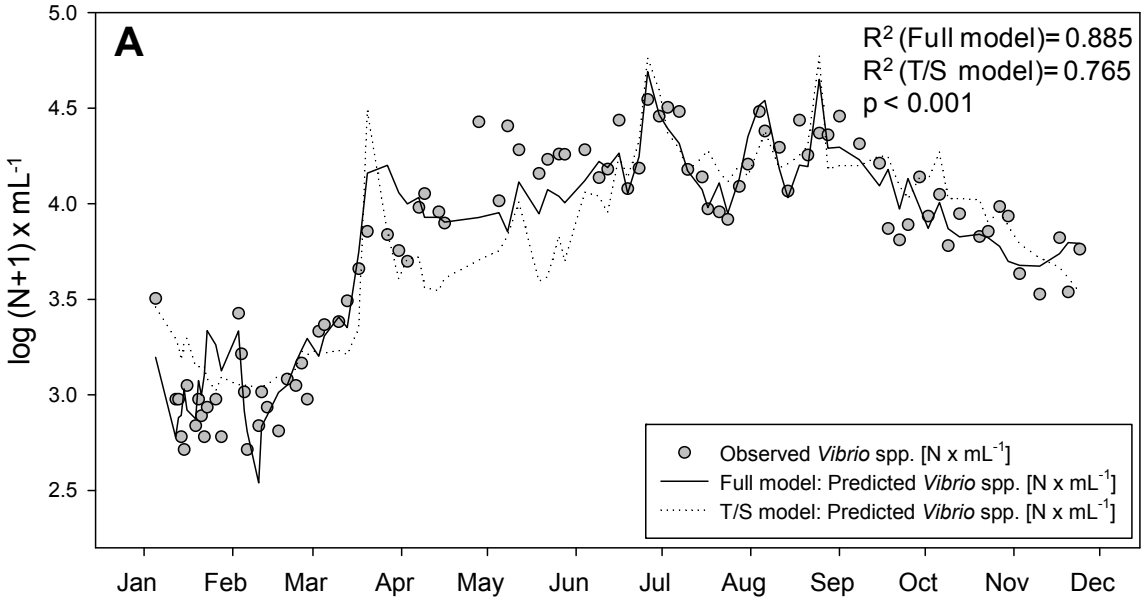
We also calculated multiple regressions with only temperature and salinity as independent variables (Table 6), two easily and rapidly measurable parameters. The multiple regression for *Vibrio* spp. abundances revealed that both, temperature and salinity had a significant influence, with salinity having the higher Beta and correlation values. Both parameters also showed a significant influence on total bacteria numbers. However, temperature had the higher influence (higher Beta and correlation values) on total bacteria, which differentiates the T/S model of total bacteria abundances from the one of *Vibrio* abundances (Table 6). In the T/S model for *Vibrio* percentages only a significant influence of salinity could be detected.

TABLE 6. Results of multiple regression analyses with temperature (T) and salinity (S) as independent variables. R- and p-values for T/S models of *Vibrio* abundances and *Vibrio* percentages and of total microbial cells are displayed. Also given are values for Beta, partial/semipartial (PK/SK) correlation, tolerance and R and p for individual variables. Significantly influencing parameters are indicated in bold ($p<0.05$).

	R^2 (model)	p (model)	Parameter	Beta	PK	SK	Tolerance	R^2 (var)	p (var)
Vibrio	**0.765**	**0.000**	**T**	**0.470**	**0.613**	**0.376**	**0.640**	**0.360**	**<0.001**
			S	**-0.508**	**-0.642**	**-0.406**	**0.640**	**0.360**	**<0.001**
%	**0.458**	**0.000**	T	-0.041	-0.045	-0.033	0.640	0.360	0.676
			S	**-0.700**	**-0.606**	**-0.560**	**0.640**	**0.360**	**<0.001**
Total	**0.781**	**0.000**	**T**	**0.736**	**0.783**	**0.589**	**0.640**	**0.360**	**<0.001**
			S	**-0.217**	**-0.349**	**-0.174**	**0.640**	**0.360**	**0.001**

The full and T/S models for *Vibrio* spp. abundances are illustrated in Figure 3A and the corresponding residuals in Figure 3B. Concurrent with this, Figure 4A and B show the full and T/S models for *Vibrio* spp. percentages and the corresponding residuals. In the full model illustration, only those variables showing a significant influence were integrated.

All multiple regression models, the full models as well as the T/S models, were significantly fitting to the observed values ($p < 0.001$). In general, the highest variances from the models could be detected in May. Higher residual values were calculated for the T/S models in comparison to the full models. When comparing the models of *Vibrio* abundances (Figure 3A/B) and percentages (Figure 4A/B), the latter ones showed larger deviations from the observed values.



***Vibrio* spp. (full model)** = 8.201 - (0.0391 * Secchi) - (0.155 * S) - (0.574 * PO_4^{3-}) – (0.634 * NO_2) + (0.111 * Diatom) + (0.152 * Dino)

***Vibrio* spp. (T/S model)** = 12.495 + (0.0480 * T) - (0.281 * S)

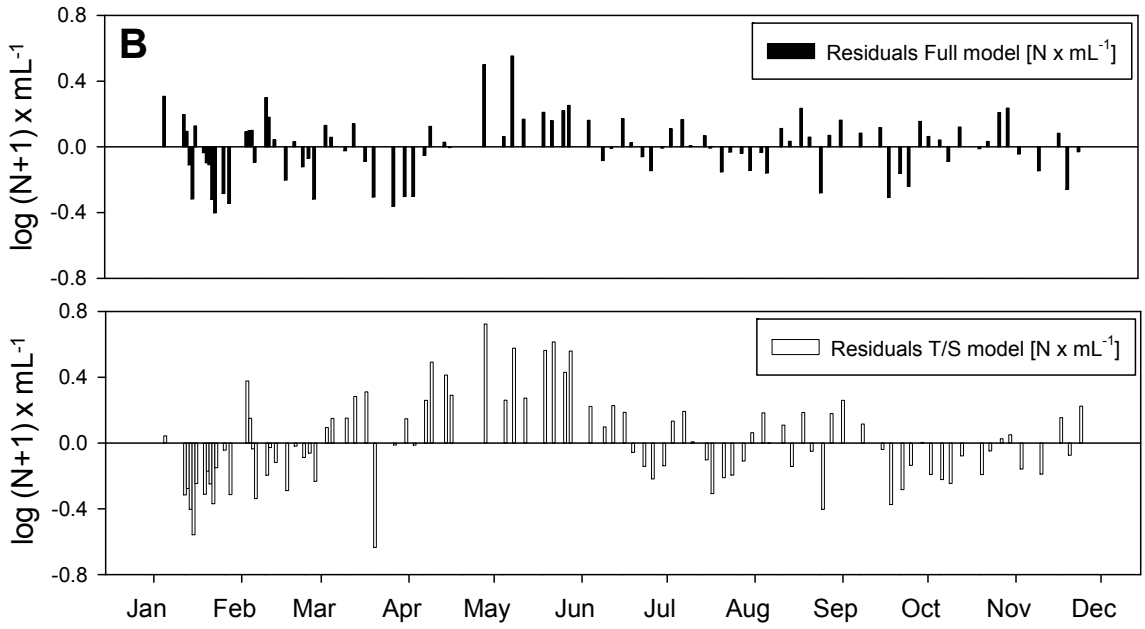
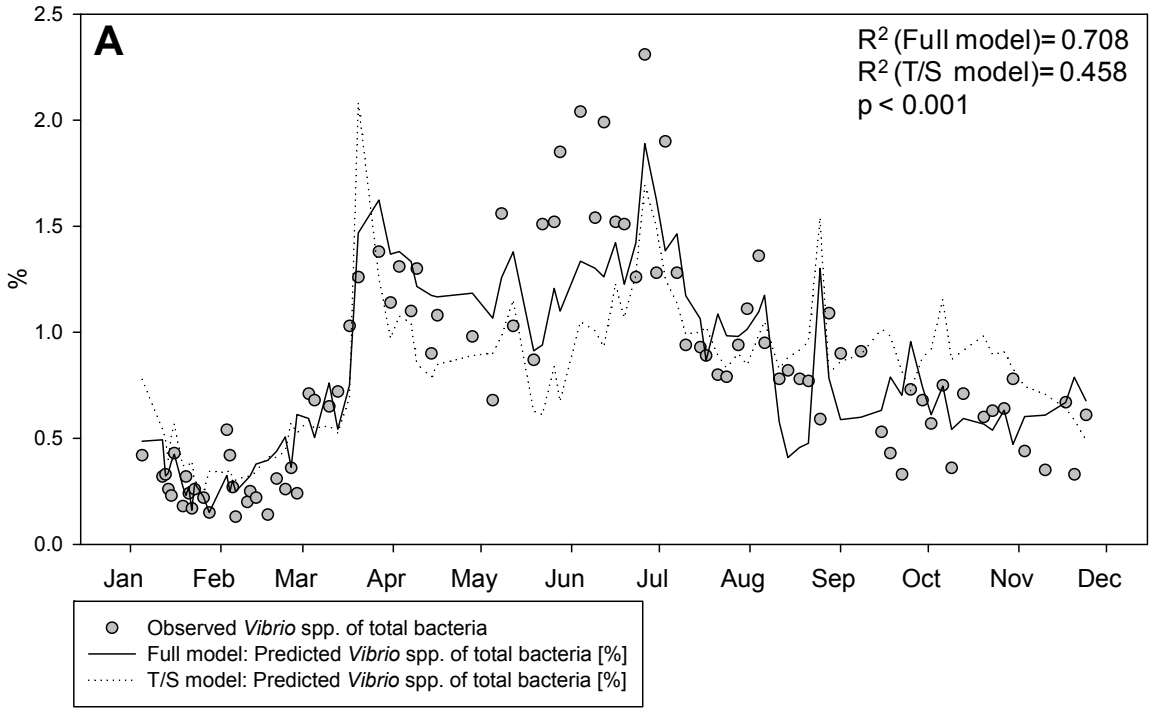


FIGURE 3. Observed and predicted *Vibrio* spp. abundances between January and December 2009 (A). Predictions are based on multiple regression analyses resulting in a full and a T/S model. Formulas are given. Corresponding residuals for full and T/S models for *Vibrio* spp. abundances are displayed (B).



% (full model) = 9.859 - (0.0373 * Secchi) - (0.258 * S) - (0.0314 * SiO_2) - (0.949 * PO_4^{3-})

% (T/S model) = 12.255 - (0.00381 * T) - (0.348 * S)

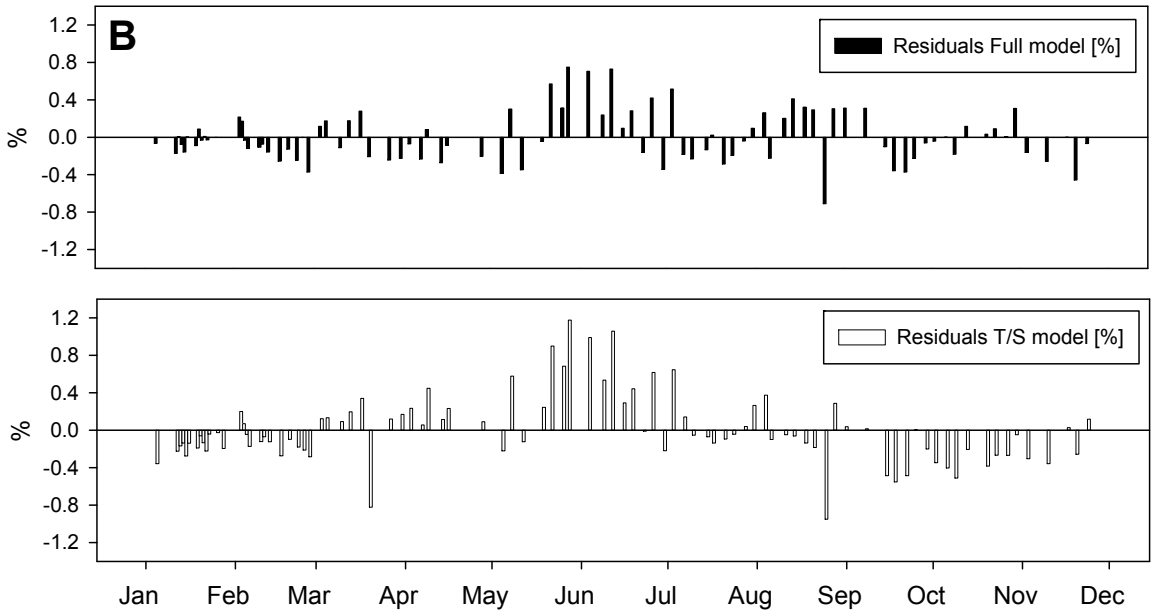


FIGURE 4. Observed and predicted *Vibrio* spp. percentages between January and December 2009 (A). Predictions are based on multiple regression analyses resulting in a full and a T/S model. Formulas are given. Corresponding residuals for full and T/S models for *Vibrio* spp. percentages are displayed (B).

Multiple Regression Analyses (seasons)

We calculated multiple regression models for each season individually. The seasons were defined as according to the astronomical seasons: summer (6/21-9/20), fall (9/21-12/20), winter (12/21-3/20), and spring (3/21-6/20).

The full model of *Vibrio* abundances in summer revealed that no single parameter had a significant influence, but dinoflagellates had the highest Beta and correlation values (Table 7). Over the whole year, nutrients showed a higher influence on *Vibrio* percentages (Table 8) than on *Vibrio* abundances.

For instance, in summer the parameter SiO_2 had a significant influence on *Vibrio* percentages. In fall the parameters NO_2 and NO_3^- and in winter the parameter secchi depth (representing water transparency) influenced *Vibrio* abundances significantly (Table 7). Although salinity did not have a significant influence, its Beta values in fall and winter were higher than those of at least one of the significantly influencing parameters. Regarding *Vibrio* percentages, the variables NO_2, PO_4^{3-} and SiO_2 had the highest Beta values in fall, but no significant p values. In winter, NO_2 and salinity influenced *Vibrio* percentages significantly. No parameter influenced *Vibrio* abundances significantly in spring, but the variable chlorophyll a showed the highest Beta and correlation values. In spring, the multiple regression model for *Vibrio* percentages was significantly influenced by the parameter secchi depth. Even though, temperature showed a higher Beta value in that model than secchi depth.

Seasonal multiple regression models were also calculated with only temperature and salinity as independent variables (Table 9). The T/S models revealed that salinity had a significant influence on *Vibrio* abundances as well as on percentages in summer; whereas temperature had a significant influence in spring. Both parameters influenced *Vibrio* spp. significantly in winter, with higher Beta and correlation values for salinity. In fall, neither temperature nor salinity showed a significant influence on the model of *Vibrio* abundances or percentages.

Except for the full and T/S models for percentages in fall, all seasonal models demonstrated a significant adaptation to the observed *Vibrio* abundances and percentages ($p < 0.05$).

TABLE 7. Results of seasonal multiple regression analyses with all measured environmental parameters as independent variables. R- and p-values for full models of *Vibrio* abundances are displayed. Also given are values for Beta, partial/semipartial (PK/SK) correlation, tolerance and R and p for individual variables. Significantly influencing parameters are indicated in bold ($p<0.05$).

Season	N	R^2 (model)	p (model)	Parameter	Beta	PK	SK	Tolerance	R^2 (var)	p (var)
Summer	24	**0.756**	**0.024**	T	-0.538	-0.341	-0.179	0.111	0.889	0.233
(6/21 - 9/20)				S	-0.102	-0.122	-0.061	0.357	0.643	0.677
				Secchi	-0.030	-0.033	-0.016	0.299	0.701	0.911
				SiO_2	0.373	0.207	0.104	0.078	0.922	0.478
				PO_4^{3-}	-0.297	-0.298	-0.154	0.268	0.732	0.301
				NO_2	0.099	0.054	0.027	0.074	0.926	0.854
				NO_3^-	0.437	0.223	0.113	0.067	0.933	0.444
				$NH4^+$	0.002	0.002	0.001	0.302	0.698	0.995
				Diatoms	0.293	0.304	0.158	0.289	0.711	0.291
				Dino	1.211	0.513	0.295	0.059	0.941	0.061
				Chla	0.053	0.052	0.026	0.237	0.763	0.860
Fall	16	**0.944**	**0.047**	T	0.238	0.218	0.053	0.049	0.951	0.678
(9/21 - 12/20)				S	-1.174	-0.682	-0.220	0.035	0.965	0.136
				Secchi	0.249	0.549	0.155	0.386	0.614	0.260
				SiO_2	0.124	0.113	0.027	0.047	0.953	0.831
				PO_4^{3-}	-0.543	-0.665	-0.210	0.150	0.850	0.149
				NO_2	**-1.427**	**-0.896**	**-0.475**	**0.111**	**0.889**	**0.016**
				NO_3^-	**0.790**	**0.837**	**0.361**	**0.208**	**0.792**	**0.038**
				$NH4^+$	0.551	0.704	0.234	0.180	0.820	0.118
				Diatoms	0.510	0.612	0.182	0.128	0.872	0.197
				Dino	0.165	0.340	0.085	0.267	0.733	0.509
				Chla	-0.773	-0.759	-0.275	0.127	0.873	0.080
Winter	31	**0.808**	**0.000**	T	-0.214	-0.265	-0.120	0.316	0.684	0.246
(12/21 - 3/20)				S	-0.901	-0.291	-0.134	0.022	0.978	0.200
				Secchi	**-0.383**	**-0.440**	**-0.215**	**0.314**	**0.686**	**0.046**
				SiO_2	-0.401	-0.237	-0.107	0.071	0.929	0.302
				PO_4^{3-}	0.010	0.015	0.006	0.428	0.572	0.950
				NO_2	-0.212	-0.353	-0.166	0.608	0.392	0.116
				NO_3^-	-0.127	-0.045	-0.020	0.025	0.975	0.845
				$NH4^+$	-0.155	-0.251	-0.113	0.537	0.463	0.273
				Diatoms	0.411	0.368	0.174	0.178	0.822	0.100
				Dino	-0.137	-0.206	-0.092	0.456	0.544	0.370
				Chla	-0.041	-0.051	-0.023	0.304	0.696	0.825
Spring	20	**0.830**	**0.041**	T	0.333	0.133	0.055	0.028	0.972	0.714
(3/21 - 6/20)				S	0.080	0.093	0.038	0.230	0.770	0.799
				Secchi	-0.075	-0.077	-0.032	0.182	0.818	0.832
				SiO_2	-0.421	-0.300	-0.130	0.095	0.905	0.400
				PO_4^{3-}	-0.184	-0.309	-0.134	0.531	0.469	0.386
				NO_2	-0.761	-0.258	-0.110	0.021	0.979	0.472
				NO_3^-	0.693	0.353	0.156	0.051	0.949	0.317
				$NH4^+$	0.039	0.044	0.018	0.218	0.782	0.905
				Diatoms	-0.013	-0.015	-0.006	0.206	0.794	0.968
				Dino	-0.183	-0.198	-0.083	0.208	0.792	0.583
				Chla	-0.829	-0.513	-0.247	0.088	0.912	0.129

TABLE 8. Results of seasonal multiple regression analyses with all measured environmental parameters as independent variables. R- and p-values for full models of *Vibrio* percentages are displayed; also given are values for Beta, partial/semipartial (PK/SK) correlation, tolerance and R and p for individual variables. Significantly influencing parameters are indicated in bold ($p<0.05$).

Season	N	R^2	p (model)	Parameter	Beta	PK	SK	Tolerance	R^2 (var)	p (var)
Summer	24	**0.773**	**0.017**	T	-0.615	-0.395	-0.205	0.111	0.889	0.163
(6/21 - 9/20)				S	0.058	0.072	0.035	0.357	0.643	0.806
				Secchi	0.058	0.066	0.032	0.299	0.701	0.822
				SiO_2	**-1.101**	**-0.543**	**-0.308**	**0.078**	**0.922**	**0.045**
				PO_4^{3-}	-0.052	-0.057	-0.027	0.268	0.732	0.847
				NO_2	0.459	0.253	0.125	0.074	0.926	0.384
				NO_3^-	-0.432	-0.228	-0.111	0.067	0.933	0.434
				$NH4^+$	-0.407	-0.424	-0.224	0.302	0.698	0.130
				Diatoms	0.034	0.038	0.018	0.289	0.711	0.897
				Dino	1.008	0.458	0.246	0.059	0.941	0.100
				Chla	-0.029	-0.030	-0.014	0.237	0.763	0.920
Fall	16	0.799	0.386	T	-0.260	-0.128	-0.058	0.049	0.951	0.810
(9/21 - 12/20)				S	-0.493	-0.202	-0.092	0.035	0.965	0.702
				Secchi	0.450	0.530	0.280	0.386	0.614	0.280
				SiO_2	1.001	0.435	0.216	0.047	0.953	0.389
				PO_4^{3-}	-1.208	-0.722	-0.467	0.150	0.850	0.105
				NO_2	-1.252	-0.681	-0.417	0.111	0.889	0.136
				NO_3^-	0.708	0.585	0.323	0.208	0.792	0.223
				$NH4^+$	0.904	0.651	0.384	0.180	0.820	0.162
				Diatoms	0.936	0.598	0.335	0.128	0.872	0.209
				Dino	0.301	0.328	0.156	0.267	0.733	0.526
				Chla	-0.689	-0.481	-0.245	0.127	0.873	0.335
Winter	31	**0.887**	**0.000**	T	-0.070	-0.116	-0.039	0.316	0.684	0.617
(12/21 - 3/20)				**S**	**-1.276**	**-0.491**	**-0.189**	**0.022**	**0.978**	**0.024**
				Secchi	-0.285	-0.429	-0.160	0.314	0.686	0.052
				SiO_2	-0.088	-0.070	-0.024	0.071	0.929	0.764
				PO_4^{3-}	-0.176	-0.323	-0.115	0.428	0.572	0.153
				NO_2	**-0.223**	**-0.460**	**-0.174**	**0.608**	**0.392**	**0.036**
				NO_3^-	-0.756	-0.333	-0.119	0.025	0.975	0.140
				$NH4^+$	-0.157	-0.324	-0.115	0.537	0.463	0.153
				Diatoms	0.351	0.403	0.148	0.178	0.822	0.070
				Dino	-0.233	-0.424	-0.157	0.456	0.544	0.056
				Chla	0.141	0.226	0.078	0.304	0.696	0.325
Spring	20	**0.858**	**0.023**	T	1.358	0.513	0.226	0.028	0.972	0.129
(3/21 - 6/20)				S	-0.032	-0.040	-0.015	0.230	0.770	0.912
				Secchi	**-0.891**	**-0.709**	**-0.380**	**0.182**	**0.818**	**0.022**
				SiO_2	-0.440	-0.338	-0.136	0.095	0.905	0.339
				PO_4^{3-}	-0.136	-0.253	-0.099	0.531	0.469	0.480
				NO_2	1.018	0.363	0.147	0.021	0.979	0.302
				NO_3^-	-0.795	-0.428	-0.179	0.051	0.949	0.217
				$NH4^+$	-0.499	-0.526	-0.233	0.218	0.782	0.119
				Diatoms	0.071	0.086	0.032	0.206	0.794	0.814
				Dino	0.018	0.022	0.008	0.208	0.792	0.951
				Chla	-0.242	-0.187	-0.072	0.088	0.912	0.604

TABLE 9. Results of seasonal multiple regression analyses with temperature (T) and salinity (S) as independent variables. R- and p-values for T/S models of *Vibrio* abundances and percentages are displayed. Also given are values for Beta, partial/semipartial (PK/SK) correlation, tolerance and R and p for individual variables. Significantly influencing parameters are indicated in bold ($p<0.05$).

	Season	N	R^2	p (model)	Parameter	Beta	PK	SK	Tolerance	R^2 (var)	p (var)
Vibrio	**Summer**	24	**0.261**	**0.042**	T (14.1 - 18.6)	0.260	0.263	0.234	0.816	0.184	0.225
					S (30.18 - 32.71)	**-0.565**	**-0.510**	**-0.510**	**0.816**	**0.184**	**0.013**
	Fall	16	**0.520**	**0.008**	T (10.9 - 16.8)	0.362	0.411	0.312	0.744	0.256	0.128
					S (31.75 - 33.69)	-0.468	-0.503	-0.403	0.744	0.256	0.056
	Winter	31	**0.581**	**0.000**	**T (3.6 - 5.5)**	**-0.378**	**-0.504**	**-0.378**	**1.000**	**0.000**	**0.005**
					S (29.19 - 34.47)	**-0.660**	**-0.714**	**-0.660**	**1.000**	**0.000**	**<0.001**
	Spring	20	**0.485**	**0.004**	**T (4.7 - 13.5)**	**0.693**	**0.695**	**0.693**	**1.000**	**0.000**	**0.001**
					S (30.64 - 33.35)	0.080	0.111	0.080	1.000	0.000	0.650
%	**Summer**	24	**0.380**	**0.007**	T (14.1 - 18.6)	-0.294	-0.319	-0.265	0.816	0.184	0.138
					S (30.18 - 32.71)	**-0.430**	**-0.443**	**-0.389**	**0.816**	**0.184**	**0.034**
	Fall	16	0.117	0.445	T (10.9 - 16.8)	-0.036	-0.033	-0.031	0.744	0.256	0.908
					S (31.75 - 33.69)	-0.359	-0.313	-0.310	0.744	0.256	0.256
	Winter	31	**0.636**	**0.000**	**T (3.6 - 5.5)**	**-0.251**	**-0.384**	**-0.251**	**1.000**	**0.000**	**0.036**
					S (29.19 - 34.47)	**-0.755**	**-0.781**	**-0.755**	**1.000**	**0.000**	**<0.001**
	Spring	20	**0.332**	**0.033**	**T (4.7 - 13.5)**	**0.554**	**0.561**	**0.554**	**1.000**	**0.000**	**0.012**
					S (30.64 - 33.35)	-0.146	-0.176	-0.146	1.000	0.000	0.472

Discussion

Vibrio spp. are autochthonous members of microbial communities in European waters and also pathogenic *Vibrio* strains are gaining in relevance in these waters. However, little is known about the distribution and driving environmental parameters of *Vibrio* spp., especially in northern European waters including the German Bight. To be able to make predictions about the dynamics of this important bacterial group harboring pathogenic species, profound investigations such as this study are essential.

Besides this study, also Eiler and coworkers quantified the *Vibrio* community in Northern European waters using the cultivation independent method 'quantitative PCR' (Eiler*, et al.*, 2006). With 4 to 96 cell x mL^{-1}, the abundances in the Baltic and Skagerrak Seas ranged far below the ones in the German Bight. The lower numbers might result from the colder water temperatures of the more northern sampling site. Alternatively, the detection yield of quantitative PCR might be lower than the one of CARD-FISH, the method used in this study.
Although GV is a probe standardly applied to quantify *Vibrio* spp. (Eilers*, et al.*, 2000, Schattenhofer*, et al.*, 2009), we confirmed its specificity before performing CARD-FISH. A coverage of 88% regarding the genus *Vibrio* implies that GV is a very reliable probe to identify *Vibrio* spp.. Species within the genus *Vibrio* not being covered by the probe are *V. cholera* and *V. mimicus*. This is not surprising, since DNA-DNA hybridization experiments revealed that both species are closely related to each other, but not to the other *Vibrio* species (Farmer & Hickman-Brenner, 2006). In recent taxonomical discussions it has even been suggested to give *V. cholera* and *V. mimicus* a distinct genus rank (Thompson, *et al*., 2004a). However, *V. cholera* and *V. mimicus* represent only an insignificant percentage of the *Vibrio* communities in northern European waters (Bauer, *et al*., 2006, Schets, *et al*., 2010). These species not having detected by the probe GV does not represent an issue considering the purpose of this study, namely to quantify *Vibrio* spp. in the German Bight.

In general, higher *Vibrio* spp. abundances occurred at periods with higher water temperatures, but the peaks of *Vibrio* spp. and temperature did not overlap completely. It is obvious that the *Vibrio* community is influenced by the water temperature, but that other parameters must have crucial effects, too.

We calculated the correlations between environmental parameters and *Vibrio* abundances / percentages using Spearman correlation analyses (Tables 3 and 4). Both variables, *Vibrio* abundances and percentages, correlated among others significantly with algal abundances and chlorophyll a. *Vibrio* spp. are positively affected by the occurrence of algae, because they can utilize the bioavailable dissolved organic substrate released by the algae. This relation between *Vibrio* spp. and algae has been described in several previous studies (Mourino-Perez*, et al.*, 2003, Eiler*, et al.*, 2006, Hsieh*, et al.*, 2008).

It has been reported for other geographical regions, that the combination and interaction of several environmental factors influences a *Vibrio* community (Hsieh*, et al.*, 2008, Turner*, et al.*, 2009). Thus, we calculated multiple regressions using all measured parameters as independent variables.

The variables which significantly influenced the full regression models were not in complete accordance with the significant parameters of the Spearman correlation analyses. The reasons are presumably the complex coherences between environmental parameters. Autocorrelation or opposing interactions might mask the effects of certain variables within the multiple regression analyses. For instance, temperature showed a highly significant positive correlation with *Vibrio* spp. in the Spearman rank analyses (Table 3), but no significant effect in the full models (Tables 5, 7). Many studies have reported that temperature is one of the main parameters influencing *Vibrio* spp. (Vezzulli, *et al*., 2009, Caburlotto, *et al*., 2010b). Also at Helgoland Roads a strong influence of temperature on *Vibrio* spp. has been detected previously (Oberbeckmann, *et al.*, in review). We know that temperature autocorrelates with many other environmental parameters (Table 4), which is supported by its very low tolerance values (Table 5). These autocorrelations might mask the actual influence of temperature on *Vibrio* spp. within the full models.

Particularly salinity had a high, significant Beta value in the full model calculation for *Vibrio* abundances. All statistical analyses in this study revealed that salinity had a strong negative correlation with *Vibrio* spp.. That implies a preference of *Vibrio* spp. for low salinity, which has also been reported elsewhere (Martinez-Urtaza, *et al*., 2008b, Baker-Austin, *et al*., 2010).

The full model for *Vibrio* percentages was significantly influenced by the nutrients SiO_2 and PO_4^{3-}. The Beta values of these parameters were negative, but most likely this does not represent a direct effect on *Vibrio* spp.. We rather assume that *Vibrio* abundances

were highest in summer, when most nutrients were already depleted by the plankton community (Figures 2 and 3). *Vibrio* bacteria can use algal released substrates very efficiently as they attach to algal cells by the formation of biofilms (Lee*, et al.*, 2003, Nakhamchik*, et al.*, 2008, Snoussi*, et al.*, 2008). This strategy and their high growth rates represent a competitive advantage over many other pelagic bacteria (Ulitzur, 1974, Aiyar*, et al.*, 2002). Therefore, the percentages of *Vibrio* spp. were particularly high at periods with decreased nutrients and increased algal abundances, respectively.

To simplify the prediction models and make them more comprehensible, we calculated multiple regressions with only temperature and salinity as independent variables. Both variables are easily measurable and are known to have a strong influence on the *Vibrio* community. As illustrated in Figures 3 and 4, the adaption of the T/S models to the observed *Vibrio* abundances and percentages was slightly poorer when compared to the full models. Nevertheless, the T/S models showed a significant adaptation. They revealed that salinity had a higher influence on the *Vibrio* abundances than temperature, while the total microbial numbers were more strongly influenced by temperature than salinity (Table 6).

To get an impression of whether the influence of environmental parameters varies over the year, multiple regressions were calculated for each season individually. Only few parameters influenced the seasonal models significantly. This statistical instability might be due to the comparably low number of data points (16 – 31 per season). However, based on the Beta and correlation values we could detect that the effects of the environmental parameters on the *Vibrio* community were not consistent but varied between the seasons. For instance, *Vibrio* abundances were primarily influenced by algal abundances and chlorophyll a in summer and spring, the blooming seasons. In fall, the parameter nutrients and in winter the parameter salinity and secchi depth (representing water transparency) were more important concerning *Vibrio* abundances.

The T/S models of the individual seasons also revealed changing influences of the parameters over the year. In spring, solely temperature had a significant influence on *Vibrio* abundances as well as on percentages (Table 9). Spring was the season during which temperature displayed a very broad range (4.7 – 13.5°C) with a variance of 8.8°C. In the remaining seasons with relatively stable temperature (variance between 2 and 6°C), salinity displayed the main influence.

The illustrations of the multiple regression models for the whole year (Figure 3A and 4A) and the corresponding residuals (Figure 3B and 4B) show that the highest variance

between observed and predicted values occurred in May. In this month a temporary increase of temperature, nutrients, diatoms and a decrease in salinity was detected (Figure 2). These changes imply a sudden change of the water body in May 2009, most likely a shift to coastal water. The sampling station of this study, Helgoland Roads, represents a highly dynamic water system. Due to its geographical location, it is influenced by northern North Sea as well as coastal waters. The *Vibrio* abundance in the coastal water obviously was greater than the abundance of *Vibrio* spp. in the more saline water body of Helgoland Roads in previous months. This is to be expected, since lower salinity, higher temperature, and algal abundances in the coastal water represent favorable conditions for *Vibrio* spp.. Besides the shifting of different water bodies at Helgoland, extreme weather events could also lead to unexpected high *Vibrio* abundances. Such events have been shown to be storms, hurricanes or weather anomalies such as El Niño, as previously described by Martinez-Urtaza and coworkers (2008a) and Wetz and coworkers (2008). Also in the North Sea an increase of storm events and wind speed has been observed (Siegismund & Schrum, 2001, Woth, *et al*., 2006) and this trend is most likely to be continued. Even though a prediction model might fit significantly with the observed *Vibrio* abundances under normal conditions, one must pay particular attention to storm events and the shifting of water bodies.
In general, all models in this study showed a significant adaption to the observed values. In common with our study, Hsieh and coworkers (2008) and de Magny and coworkers (2009) described T/S models as a comprehensible and reliable way to assess *Vibrio* abundances. Data of temperature and salinity alone can give an impression about *Vibrio* concentration in the water and can indicate periods where *Vibrio* spp. monitoring is necessary. This could reduce the amount of sampling and laboratory work tremendously. For a more detailed analysis of the response of *Vibrio* spp. to environmental parameters or the assessment of the contribution of *Vibrio* spp. to the whole bacterial community at certain environmental conditions or seasons, full model calculation is a more suitable tool.

So far, there are only few data available concerning the complex influences of environmental parameters on *Vibrio* spp. in northern European waters. This work contributes tremendously to understand the dynamics of *Vibrio* communities in these regions. High water temperatures, low salinity, high algae abundances, the shifting of coastal waters or storm events favor the increase of *Vibrio* spp. and require particular

attention. It seems to be a suitable next step to get comparable insights on the species or even strain level. *In situ* techniques targeting individual genes such as GeneFISH or RING-FISH might be reliable and effective tools to do so (Zwirglmaier*, et al.*, 2004, Moraru*, et al.*, 2010).

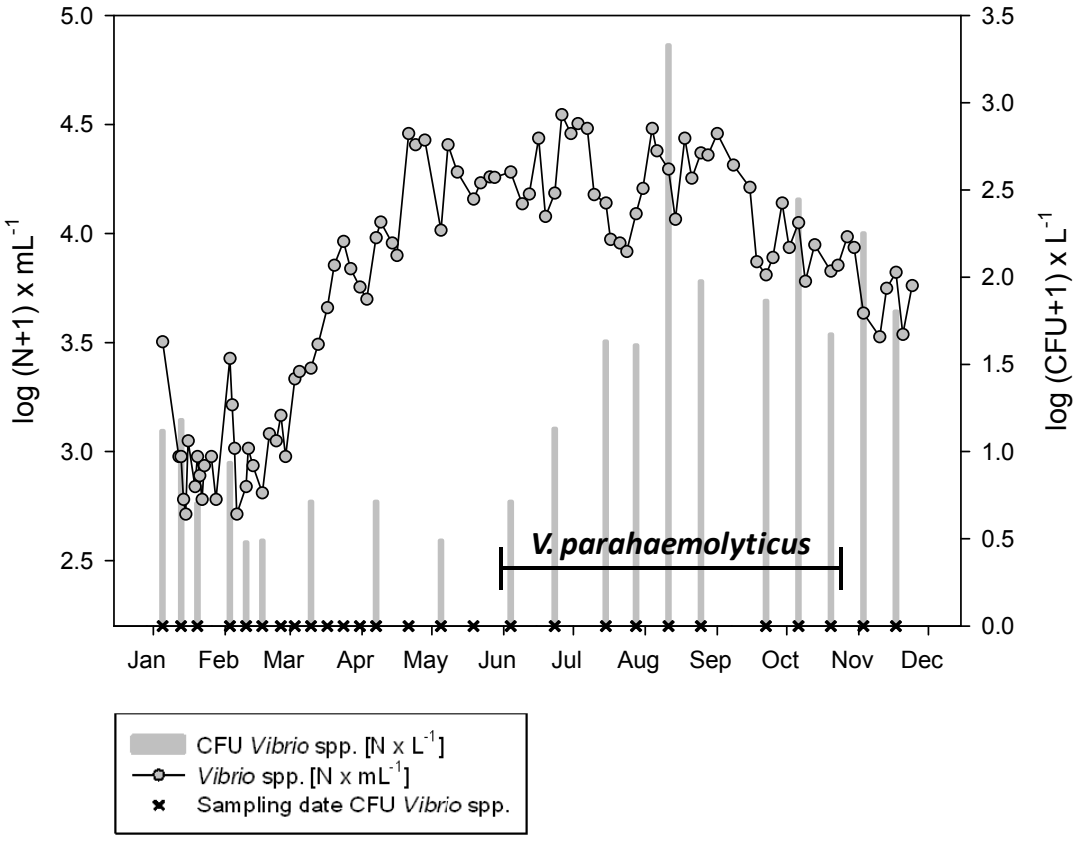


FIGURE 5. *Vibrio* spp. abundances quantified using CARD-FISH (scatter plot) and cultivation on TCBS agar (bar chart) between January and December 2009. Also indicated is the period, when *V. parahaemolyticus* was detected using a cultivation approach.

In our previous study using a cultivation approach, we observed a strong correlation between the potentially pathogenic species *V. parahaemolyticus* and water temperature (Oberbeckmann, *et al.*, in review). Pathogenic strains of *V. parahaemolyticus* can cause foodborne gastroenteritis and have been reported increasingly in European waters over the last decade. We know that *V. parahaemolyticus* could be detected between June and October 2009 at Helgoland Roads (Figure 5) and that the occurrence of *V. parahaemolyticus* was concurrent with warmer water temperatures (> 15°C). In the course of a method evaluation we observed that *V. parahaemolyticus* strains grow distinctly faster with increasing incubation temperature (up to 37°C). This is supported by other studies, which also revealed increased growth of *V. parahaemolyticus* as a

function of temperature (Nishina*, et al.*, 2004, Burnham*, et al.*, 2009). Previous studies have further reported that pathogenicity of *V. parahaemolyticus* is increased with higher temperature (Martinez-Urtaza, *et al*., 2008b, Mahoney, *et al*., 2010, Rodriguez-Castro, *et al*., 2010). These findings are alarming in the light of climate change.
Further investigations of the *Vibrio* community and other bacterial communities containing potentially pathogenic species are urgent, also in temperate waters such as the coastal shelf area of the North Sea.

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GENERAL DISCUSSION

Bacteria of the genus *Vibrio* are ubiquitous in European waters. However, human pathogenic *Vibrio* strains have been detected infrequently in European waters, but over the last decade they seem to have increased. For instance, reports of wound infections caused by *Vibrio* species, such as *V. vulnificus*, have become more frequent in the Baltic Sea (Table 1).

TABLE 1. Wound infections in Europe caused by *Vibrio* spp. due to contact with contaminated water (according to Agency of Risk Assessment Berlin, Germany; Strauch, 2010)

Year	Country	Species	Number of cases	Infection source
1994	Germany	*V. vulnificus*	1	Baltic Sea
	Denmark	*V. vulnificus*	11	Seawater
	Sweden	*V. vulnificus*	1	Baltic Sea
2002	Germany	*V. vulnificus*	1	Baltic Sea
2003	Germany	*V. vulnificus*	2	Baltic Sea
2004	Sweden	*V. cholerae* non-O1 / non-O139	1	Baltic Sea
2006	Germany	*V. vulnificus*	3	Baltic Sea
	Denmark	*V. alginolyticus*	7	Baltic Sea
		V. parahaemolyticus	7	Baltic Sea
		V. alginolyticus	1	
	Sweden	*V. cholerae* non-O1 / non-O139	3	Baltic Sea
	Poland	*V. cholerae* non-O1 / non-O139	2	Lake
	The Netherlands	*V. alginolyticus*	3	North Sea

In recent years, also pathogenic *V. parahaemolyticus* strains emerged in European waters, for instance in Spain, Italy and France (Martinez-Urtaza*, et al.*, 2005, Quilici*, et al.*, 2005, Ottaviani*, et al.*, 2008). These diarrhea causing strains carry specific virulence factors and have previously led to severe epidemics worldwide. But thus far, profound investigations on the pathogenicity, distribution and ecology of *V. parahaemolyticus*, especially in northern European waters, are lacking. From a single study we know that *V. parahaemolyticus* is abundant in the German Bight (Lhafi & Kühne, 2007), but further investigations are required. Any information on the abundance, pathogenicity and response to environmental conditions of *Vibrio* communities including *V. parahaemolyticus* provides a valuable insight into understanding the dynamics of this important microbial group in northern European waters. Therefore, *Vibrio* spp. at Helgoland Roads (North Sea, Germany) were studied elaborately in this thesis, with special emphasis on *V. parahaemolyticus*.

The research aims of this thesis were the following four main points, all with special emphasis on the potentially pathogenic species *V. parahaemolyticus*: (1) to evaluate a wide range of methods and define a reliable approach to characterize *Vibrio* spp.; (2) to quantify *Vibrio* spp. at Helgoland Roads; (3) to characterize the *Vibrio* community at Helgoland Roads; (4) to analyze the effects of environmental parameters on *Vibrio* spp. abundances and population structures.
In the following, the aims and results of this thesis are discussed in a general context.

Method evaluation

A large number of methods have been described for the characterization of *Vibrio* species. Currently, an international team of scientists aims to define standardized and worldwide accepted methods for the investigation of *Vibrio* spp. (Leonard*, et al.*, 2009). The selected methods should be highly reliable, cost- and labor-effective and applicable on a global scale. One of the central points in this context is the detection of *V. parahaemolyticus*, one main cause for foodborne gastroenteritis worldwide.
The method evaluation accomplished in this thesis defines suitable tools for the characterization of *V. parahaemolyticus* and might be the first step towards a standardized approach for the classification of this species.
To identify *Vibrio* isolates, *rpoB* gene sequencing is reported as very reliable tool (Ki*, et al.*, 2009). This thesis confirmed the usefulness of *rpoB* gene sequencing as basis for phylogenetic analyses of *Vibrio* isolates (Oberbeckmann*, et al.*, 2011). Using *rpoB* gene sequencing, even closely related species such as *V. parahaemolyticus* and *V. alginolyticus* could be differentiated. To identify *V. parahaemolyticus* using specific marker genes, several protocols have been suggested in the literature (Xie, *et al*., 2005, Bauer & Rørvik, 2007, Croci, *et al*., 2007b, Drake, *et al*., 2007). PCR approaches which target either the gene *tlh* or the gene *toxR* are most frequently applied. The results of this thesis reveal that *toxR* is a more specific marker for *V. parahaemolyticus* than *tlh*, since also several *V. alginolyticus* strains were proven positive for *tlh*. Some *V. parahaemlyticus* strains represent a threat to humans, which makes it urgent to investigate the pathogenicity of identified *V. parahaemolyticus* isolates. The most common method for doing so is the application of a multiplex-PCR targeting the virulence-associated genes *tdh* and *trh*. We showed that a PCR-protocol published by the U.S. Food and Drug Administration (DePaola & Kaysner, 2004) is very specific and reliable to detect *tdh* / *trh*. Our findings confirmed the results of Croci et al. (2007a).

Even though none of the *Vibrio* isolates from Helgoland carried *tdh*, we found some *V. parahaemolyticus* isolates to be hemolytic on blood agar. This observation suggests that other factors besides *tdh* are involved in the hemolytic activity of *V. parahaemolyticus*. This finding is supported by other studies (Bej*, et al.*, 1999, Izutsu*, et al.*, 2008). Additional marker genes for the pathogenicity of *V. parahaemolyticus* have been recently suggested by Caburlotto *et al*. (2009).

Continued investigations are important to improve and develop protocols to estimate the pathogenicity of *V. parahaemolyticus*, especially in times of spreading pandemic strains. Currently, the detection of species-specific and virulence-associated genes of *V. parahaemolyticus* is based on cultivated or enriched sample material. In the field of medical microbiology, cultivation-independent applications are becoming more and more relevant, because they allow an unbiased insight into the abundance of single species or strains. Due to their low quantity compared to total bacteria in the environment, it is difficult to detect *V. parahaemolyticus* in environmental samples directly without enrichment (Dileep*, et al.*, 2003, Drake*, et al.*, 2007, Robert-Pillot*, et al.*, 2010). A future task would be to develop highly sensitive and reliable techniques to identify and quantify *V. parahaemolyticus* and its pathogenicity, without cultivation or enrichment.

Quantification

In the course of this thesis, *Vibrio* spp. at Helgoland Roads were quantified using a cultivation as well as a cultivation-independent approach. The cultivation-independent approach aimed to capture the whole *Vibrio* community using FISH, which had not been carried out extensively for North Sea water before. The *Vibrio* numbers were detected by the probe GV (Giuliano*, et al.*, 1999), which is applied as standardized probe to quantify *Vibrio* spp. (Eilers*, et al.*, 2000, Wietz*, et al.*, 2010).

The lowest *Vibrio* abundances were determined in January and February, whereas the highest numbers occurred between late April and early September. Numbers of up to 3.37×10^4 cells x mL^{-1} were detected, representing up to 2.22% of the total bacteria. Since *Vibrio* bacteria have previously not been subject to profound cultivation-independent investigations in the North Sea, comparable *Vibrio* numbers from other studies are lacking.

We also applied a cultivation approach to quantify only potentially pathogenic *Vibrio* spp., using selective medium and temperature. In doing so, free-living *Vibrio* spp. up to

4.3 x 10^3 N x L^{-1} were counted. Likewise, the detected plankton-attached *Vibrio* spp. followed the same trend as free-living *Vibrio* spp.. This implies that *Vibrio* bacteria, whether they are attached or free-living, are mainly influenced by the same parameters throughout the year. In a study on Italian waters, Maugeri and coworkers (2004) found *Vibrio* numbers comparable to the ones at Helgoland Roads. Due to the higher water temperatures, one would expect higher *Vibrio* abundances in the Mediterranean Sea than in the North Sea. Potentially, the exceptionally high salinity (up to 38) at the Italian sampling station counteracted the positive effect of the warm water temperature on the *Vibrio* community.

Considering shellfish-associated *Vibrio* spp. at Helgoland Roads, numbers up to 240 MPN x g^{-1} were detected. Other investigations in the North Sea (Oosterschelde, the Netherlands) (Schets, *et al*., 2010) revealed MPN values of *Vibrio* in shellfish similar to or slightly below the ones detected at Helgoland Roads. But compared to the numbers of *Vibrio* spp. in shellfish in the warmer waters of São Paulo State (Brazil) and Florida (USA) (Ellison*, et al.*, 2001, Sobrinho*, et al.*, 2010), the *Vibrio* numbers in shellfish in the North Sea are distinctly lower.

The governmental regulations concerning *Vibrio* spp. in seafood vary from species to species. According to the U.S. Food and Drug Administration (USFDA), seafood is to be removed from the market, when the presence of any pathogenic strain of *V. cholerae* or *V. vulnificus* is detected (USFDA, 2001a). The critical threshold for *V. parahaemolyticus* cells is 1 x 10^4 N x g^{-1}, whether pathogenicity factors are present or not. In the UK, the infection hazard is defined as high when > 1 x 10^3 CFU *V. parahaemolyticus* per gram ready-to-eat food are detected (Health Protection Agency, 2009). Based on these critical levels, the occurrence of *Vibrio* spp. at Helgoland Roads indicates no threat to humans so far. In Germany, no food or bathwater monitoring concerning *Vibrio* spp. is mandatory. But since *Vibrio* bacteria benefit from the effects of climate change, regular quantification of *Vibrio* spp. in the North Sea is highly recommended. The creation of a well-funded data base would make it possible to detect iterative seasonal dynamics of *Vibrio* abundances and observe an increase or decrease in *Vibrio* spp. numbers over a longer time scale.

Characterization

An aim of this thesis was also the characterization of the *Vibrio* community with special emphasis on the potential pathogen *V. parahaemolyticus*. During a 2-year sampling period, *Vibrio* spp. were isolated from seawater, plankton and shellfish. The cultivation was performed using specific TCBS agar and an incubation temperature of 37°C to selectively cultivate potentially human pathogenic strains. We established a culture collection and classified > 200 *Vibrio* isolates using the polyphasic approach defined in this thesis (Oberbeckmann*, et al.*, 2011).

The phylogenetic analyses based on *rpoB* gene sequencing revealed *V. alginolyticus* to be the dominant *Vibrio* species at Helgoland Roads (considering the culturable *Vibrio* bacteria growing at 37°C). The second most abundant *Vibrio* species at Helgoland Roads turned out to be *V. parahaemolyticus*. Few isolates were assigned to the species *V. harveyi*. A similar *Vibrio* species composition has also been reported in other northern European waters (Hervio-Heath, *et al*., 2002, Lhafi & Kühne, 2007, Schets, *et al*., 2010). In geographical regions featuring warmer water temperatures, the potentially pathogenic species *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* have been shown to be more relevant (Su & Liu, 2007, Eja*, et al.*, 2008, Cabral, 2010).

To investigate the intraspecific diversity of the potential pathogen *V. parahaemolyticus* and its close relative *V. alginolyticus*, we applied genomic fingerprinting. For these two species, ERIC-fingerprinting revealed three distinct groups at Helgoland Roads. One group represented *V. parahaemolyticus* strains, the second one *V. alginolyticus* strains. Strains from the third group showed high phylogenetic similarity to reference sequences of *V. alginolyticus*, based on *rpoB* gene sequencing. However, the third group represented a distinct cluster between *V. alginolyticus* and *V. parahaemolyticus*. This cluster might be a transition group between *V. parahaemolyticus* and *V. alginolyticus*, harboring genomic characteristics of both species. In recent years, improvements to the classical systematic model for bacteria have been suggested (Cohan, 2009). Strains within the same species have been shown to share less than 50% of their proteins, based on very different genome structures (Welch*, et al.*, 2002). The adaptation to different environmental conditions, different life strategies and horizontal gene transfer (HGT) can lead to various ecotypes of one species (López-López*, et al.*, 2005, Polz*, et al.*, 2006, Bailly*, et al.*, 2007). So far, these different ecotypes have generally gone unrecognized and were not an element of systematic investigations. Tracking the development and establishment of ecotypes would enable microbial ecologists to gain

valuable insights into genome evolution and population diversity. Also *Vibrio* bacteria show high genomic polymorphism and intraspecies variability and are subject to frequent HGT (Hazen*, et al.*, 2010). The formation of different ecotypes has already been reported for *V. vulnificus* (Rosche*, et al.*, 2010). The *Vibrio* transition group from Helgoland Roads might display a *V. alginolyticus* ecotype, representing a genomic transition to *V. parahaemolyticus*, the cause of serious diarrhea.

So far, no pathogenicity factors such as the genes *tdh* / *trh* could be detected in *Vibrio* strains at Helgoland Roads. Several European studies, however, describe water and especially sediments to be a reservoir for pathogenicity vectors and potentially pathogenic *V. parahaemolyticus* (Ellingsen, *et al*., 2008, Vezzulli, *et al*., 2009, Deter, *et al*., 2010b). A change of environmental conditions might cue the spread of virulence factors. Due to frequent HGT of *Vibrio* bacteria, non-pathogenic *V. parahaemolyticus* strains can easily obtain virulence factors on plasmids, as part of phage RNA or in terms of genomic islands. The transfer of toxin genes from pathogenic to non-pathogenic strains by phages has already been described for the species *V. cholerae* (Choi, *et al*., 2010a). Moreover, the increased import of seafood to Europe represents a platform for the introduction of pandemic strains.

Just recently the pandemic clone *V. parahaemolyticus* O3:K6 was introduced to Spain, Italy and France (Martinez-Urtaza*, et al.*, 2005, Quilici*, et al.*, 2005, Ottaviani*, et al.*, 2008). One might speculate that the first finding of *V. parahaemolyticus* O3:K6 will be reported for the North Sea in the near future.

Estimation of effects of environmental parameters

Due to their high genome plasticity, *Vibrio* bacteria can respond rapidly to environmental changes. This thesis aimed to elucidate the response of *Vibrio* spp. to environmental parameters at Helgoland Roads.

A wide range of environmental parameters, such as temperature, salinity and phytoplankton occurrence, have already been shown to influence *Vibrio* communities on a global scale (Hsieh*, et al.*, 2008, Turner*, et al.*, 2009, Vezzulli*, et al.*, 2009). However, only very few studies concerning the ecology of *Vibrio* communities have been carried out in northern European waters, such as the North Sea. The dynamics of *Vibrio* communities in the North Sea are therefore not well understood and predictions are impossible.

Our seasonal investigations of *Vibrio* abundances revealed that the main influencing parameters vary throughout the year. As described for other geographical regions, especially high temperature, high phytoplankton abundance and low salinity favor high *Vibrio* abundances at Helgoland Roads. We further observed a link between increased *Vibrio* abundances and the shifting from coastal water towards Helgoland. Helgoland Roads is a highly dynamic water system, which is frequently affected by the shifting of northern North Sea and coastal waters. Besides the shifting of water bodies, also storm events can lead to exceptional high *Vibrio* abundances at Helgoland Roads, as described for other regions (Wetz*, et al.*, 2008, Lara*, et al.*, 2009). In recent years, an increase of water temperature as well as of storm events has been reported for the German Bight (Siegismund & Schrum, 2001, Wiltshire*, et al.*, 2010), and an increase of *Vibrio* abundances, including potentially pathogenic species, can be expected.

Considering the potentially pathogenic species *V. parahaemolyticus*, the risk for infections is highest in summer months when the water temperature reaches its peak. At Helgoland Roads, most *V. parahaemolyticus* strains were cultivated in August at water temperatures above 18°C. High temperatures, combined with events of low salinity, have also been reported to be linked to increased abundances of this potential pathogen elsewhere (Martinez-Urtaza, *et al*., 2008b, Deter, *et al*., 2010b, Sobrinho, *et al*., 2010).

Based on this thesis we learned more about the influence of environmental parameters on the population structure of *V. parahaemolyticus* in the German Bight. We identified a complex combination of parameters which structure the *V. parahaemolyticus* population at Helgoland Roads. The strongest influence was shown for the parameters temperature, salinity and NO_3^-, but also dinoflagellates, SiO_2, secchi depth (representing water transparency), and NO_2 had a distinct influence on the structure of the *V. parahaemolyticus* population. To go even more into detail, previous studies investigated the effects of environmental parameters on the toxicity of *V. parahaemolyticus*. Rodriguez-Castro, *et al.* (2010) detected a correlation between water temperature and the presence of the genes *tdh* and *trh*. Another study revealed that the expression of some virulence-associated characteristics such as hemolysin, cytotoxicity, biofilm formation or motility is correlated with increased temperature (Mahoney*, et al.*, 2010). In consequence, rising water temperatures will most likely not only lead to increasing *V. parahaemolyticus* abundances, but to a higher percentage of pathogenic *V. parahaemolyticus* strains in temperate waters.

Also outbreaks caused by another *Vibrio* pathogen, *V. cholerae*, have been proven to be closely linked to climate variability (Koelle, 2009). In 1991, cholera reemerged in South America causing several epidemics. Pathogenic as well as non-pathogenic *V. cholerae* strains were detected at the coast of South America previous to 1991, but didn't cause disease outbreaks up to that point. Most likely a change of environmental conditions triggered the *V. cholerae* numbers past a threshold value, resulting in the disease outbreaks (Lipp*, et al.*, 2002).

The effects of climate change are increasingly in evidence in the North Sea, which represents a highly dynamic European water body. Ongoing observations of *Vibrio* spp. and the change of environmental parameters, which might affect pathogenic *Vibrio* species, are therefore crucial in these waters.

Conclusions

This thesis represents a first and invaluable step towards the understanding of the occurrence and ecology of *Vibrio* spp., with special emphasis on *V. parahaemolyticus*, within the temperate model ecosystem of the North Sea.

Vibrio numbers up to 3.37 x 10^4 cells x mL^{-1} were detected at Helgoland Roads using a cultivation-independent approach. Targeting solely potentially pathogenic *Vibrio* spp., numbers up to 4.3 x 10^3 N x L^{-1} (water, plankton) and 240 MPN x g^{-1} (shellfish) were quantified using a selective cultivation approach. These abundances are not alarming so far, but they range slightly above the *Vibrio* counts in other European waters.

A reliable, cost- and labor-effective approach was defined to characterize *Vibrio* spp. with special emphasis on the potentially human pathogenic species *V. parahaemolyticus* (Oberbeckmann*, et al.*, 2011). This hierarchical approach includes (i) the sequencing of the *rpoB* gene (encoding the RNA polymerase β-subunit) to differentiate even closely related *Vibrio* species, (ii) the use of the *toxR* gene as specific marker for *V. parahaemolyticus*, (iii) the screening for the virulence-associated genes *tdh* and *trh*, and (iv) the performance of genomic fingerprinting using ERIC-PCR to identify the intraspecies variability of *V. parahaemolyticus* and its close relative *V. alginolyticus* at Helgoland Roads.

Applying this analysis scheme, we gathered insights into the *Vibrio* community at Helgoland Roads, focusing on the *V. parahaemolyticus* / *V. alginolyticus* populations (Oberbeckmann, *et al*., in review). The large majority of *Vibrio* strains isolated from seawater, plankton and shellfish at Helgoland Roads were identified as *V. alginolyticus*,

followed by the species *V. parahaemolyticus*. Only few strains were classified as *V. harveyi* or other *Vibrio* species. None of the *V. parahaemolyticus* strains harbored the virulence-associated genes *tdh* or *trh*, although several strains were proven positive for hemolytic activity. Future studies should include other marker genes involved in the pathogenicity mechanism of *V. parahaemolyticus* (Izutsu*, et al.*, 2008, Caburlotto*, et al.*, 2009). Genomic fingerprinting of *V. parahaemolyticus* and *V. alginolyticus* strains revealed three distinct groups at Helgoland Roads. One group represented *V. parahaemolyticus* strains, the second one *V. alginolyticus* strains and the third one *V. alginolyticus* strains with genomic attributes of *V. parahaemolyticus*. The third group displays the high genomic plasticity and intraspecific variability of *Vibrio* spp. and represents an exciting research object for further studies.

Due to their high genome plasticity, *Vibrio* bacteria are able to respond rapidly to environmental changes. The environmental parameters which most influence the *Vibrio* community at Helgoland Roads and which structure the *V. parahaemolyticus* population were identified. High temperature especially combined with events of low salinity and high abundances of phytoplankton led to increased *Vibrio* numbers at Helgoland Roads (Oberbeckmann, *et al*., submitted). The extent of the influence of the parameters, however, varies strongly between seasons. The results also revealed that the *V. parahaemolyticus* population at Helgoland Roads is structured by a complex combination of parameters, including temperature, NO_3^-, salinity, dinoflagellates, SiO_2, secchi depth and NO_2.

This thesis represents a pilot study on *Vibrio* spp. in the German Bight, with special emphasis on *V. parahaemolyticus*. It is the first work in these waters to extensively investigate the *Vibrio* community. Important knowledge of abundance, community composition, pathogenicity and ecology of *Vibrio* spp., and in particular of *V. parahaemolyticus*, was gained. The obtained insights will help tremendously to estimate the distribution and dynamics of *Vibrio* communities, including *V. parahaemolyticus,* in Northern European waters, such as the North Sea.

SUMMARY

Pathogenic *Vibrio* strains represent an increasing health issue in European waters. For instance, in 2004 the spreading of the pandemic strain *V. parahaemolyticus* O3:K6 to Europe was reported, after it has caused severe epidemics worldwide. Even though, profound investigations on *Vibrio* spp. including *V. parahaemolyticus* are lacking, especially in northern European waters.

This thesis represents a pilot study for the German Bight, investigating the *Vibrio* community extensively, with special emphasis on *V. parahaemolyticus*. Insights into abundance, community composition and response to environmental parameters of *Vibrio* spp. at Helgoland Roads (North Sea, Germany) were obtained.

Using a cultivation-independent approach, *Vibrio* numbers up to 3.37 x 10^4 cells x mL^{-1} were detected at Helgoland Roads. Targeting solely potentially pathogenic *Vibrio* spp., numbers up to 4.3 x 10^3 N x L^{-1} (water, plankton) and 240 MPN x g^{-1} (shellfish) were quantified using a selective cultivation approach.

No standardized approach for the investigation of *Vibrio* spp. exists, but many different characterization methods have been suggested. In the course of this thesis, a broad range of microbiological and molecular biological methods have been evaluated regarding the usefulness for the investigation of *Vibrio* spp. and the potentially pathogenic species *V. parahaemolyticus*. A reliable, cost- and labor-effective approach was defined including (i) the sequencing of the *rpoB* gene (encoding the RNA polymerase β-subunit) to differentiate even closely related *Vibrio* species, (ii) the use of the *toxR* gene as specific marker for *V. parahaemolyticus*, (iii) the screening for the virulence-associated genes *tdh* and *trh*, and (iv) the performance of genomic fingerprinting using ERIC-PCR to identify the intraspecies variability of *V. parahaemolyticus* and its close relative *V. alginolyticus* at Helgoland Roads.

Applying this polyphasic approach, we gathered insights into the *Vibrio* community at Helgoland Roads, focusing on *V. parahaemolyticus* and *V. alginolyticus*. *Vibrio* strains. were isolated from seawater, plankton and shellfish using selective conditions to target solely the potentially human pathogenic strains. The species *V. parahaemolyticus* appeared to be the second most abundant species at Helgoland Roads, behind its close relative *V. alginolyticus*. None of the *V. parahaemolyticus* strains carried the virulence-associated genes *tdh* or *trh*, but several strains were proven positive for hemolytic activity. Genomic fingerprinting revealed insights into the intraspecific diversity of *V parahaemolyticus* and *V. alginolyticus* strains at Helgoland Roads. Based on the

fingerprints, three distinct groups became evident. One group represented *V. parahaemolyticus*, the second one *V. alginolyticus*. The third group was located in between the two species, displaying the high genomic plasticity and intraspecific variability of *Vibrio* spp..

To be able to estimate the distribution and dynamics of *Vibrio* spp. in response to environmental conditions, we assessed the effects of environmental parameters on the *Vibrio* community at Helgoland Roads. High temperature in combination with events of low salinity and high abundance of phytoplankton led to increased *Vibrio* numbers at Helgoland Roads. The influence of the parameters, however, varied between the seasons. The population of the potentially pathogenic species *V. parahaemolyticus* at Helgoland Roads appeared to be structured by a complex combination of parameters, including temperature, NO_3^-, salinity, dinoflagellates, SiO_2, secchi depth and NO_2. Changing environmental conditions, especially the ongoing rise of water temperatures, might not only lead to increasing numbers of *Vibrio* spp. in the German Bight, but also to a community shift towards potentially pathogenic species such as *V. parahaemolyticus*.

Being the first profound study on *Vibrio* spp. in the German Bight, this thesis represents an invaluable first step towards the understanding of this important microbial group, harboring the potential pathogen *V. parahaemolyticus*, in northern European waters.

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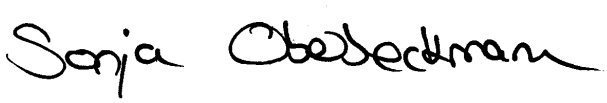
DECLARATION

I herewith declare that this thesis is my own work and effort and has been written independently. Where other sources of information have been used, these have been cited and are listed in the references. Furthermore, I declare that this work has not been submitted to any other university for the conferral of a degree.



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