Pathogenic Vibrio spp. in Northern European Waters

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Preface

Global warming and associated changes in local climate are projected to affect precipitation and droughts. On this account, water resources may be eminently at risk towards the impacts of climate change. Therefore the German Federal Ministry of Transport, Building and Urban Development (BMVBS) launched the interdisciplinary research programme KLIWAS (“Impacts of Climate Change on Waterways and Navigation”) in order to investigate potential impacts of climate change on German waterways so as to further guarantee and develop their performance and use as well as to propose adaptation options. Joined institutes are the four supreme federal agencies: Federal Waterways Engineering and Research Institute (BAW), German Maritime and Hydrographic Agency (BSH), National Meteorological Service (DWD) and Federal Institute of Hydrology (BfG).

One of the 30 projects within KLIWAS deals with the potential effect of climate change on waterborne infectious diseases and the hygienic state of German coastal waterways and estuaries (project 3.04 “Impacts of climate change on microbial water quality and their implications for dredged material management in coastal waters” carried out by BfG). Heavy rainfall and run-off for example can modify transport and distribution of microbial pathogens, and temperature may affect their survival and growth. Hence, changes in climate may increase the risks of water-borne diseases and alter their geographic range.

Pathogenic *Vibrio* spp. are the main model organisms of this KLIWAS-project and belong to the natural marine flora in estuary and seawater environments worldwide. The genus *Vibrio* contains at least 12 pathogenic bacteria species, which could cause primary (consumption of raw seafood) or secondary septicaemia (infection of pre-existing superficial wounds). As high temperatures promote *Vibrio* spp. growth, these infections have so far mainly been a problem in the tropics and subtropics. In recent years however, case reports have been increasing in the temperate Seas of Northern Europe, particularly during heat wave episodes. Although particularly the number of bathing water-related cases is small so far, concern is growing that *Vibrio*-related infections may become an emerging disease in these areas as a consequence of climate change. Along the German Baltic Sea coast human infections due to *Vibrio vulnificus* are known since 1994. For a shorter time, sporadic cases of *Vibrio* infections have also been recorded for the more saline North Sea waters. A continuing trend towards higher water temperatures may increase these severe infections.

However, the ecology of vibrios in Northern European waters is not well studied at the moment and a cross-border communication network of scientists involved in this research field is still lacking. With this 1st international symposium, we aim at creating an international network of researchers, to exchange knowledge and information concerning pathogenic vibrios in Northern European Waters, to bundle activities and thus to strengthen *Vibrio* research in these areas. Focus of the symposium will mainly be bathing water-related issues and interactions of vibrios with their environment, including overviews over past and present data, methodologies, risk assessment, state and gaps of knowledge, and future research tasks.
We would like to thank cordially all those who have contributed to this symposium be it in terms of oral presentations, posters or lively discussion. Special thanks go to Mrs. Yvonne Strunck, BfG, for her assistance in organising this symposium. We also thank the BMVBS for funding this symposium through the research programme KLIWAS.
Effect of global warming on *Vibrio* spp.
in the temperate marine environment

**Luigi Vezzulli, Ingrid Brettar, Elisabetta Pezzati, Philip C. Reid, Rita R. Colwell, Manfred G. Höfle and Carla Pruzzo**

1 Introduction

Vibrios are still regarded by most marine microbiologists as the dominant culturable bacteria in the ocean and represent an important cause of morbidity and mortality in humans and marine animals throughout the world. The 2010-2011 MCCIP Annual Report Card (www.mccip.org.uk/arc) that provides an up-to-date assessment of how climate change is affecting UK seas considered, for the first time, the potential future increases in marine vibrios as an emergent issue (also based on the occurrence of an unprecedented increase in the number of bathing infections that have been associated with warm water *Vibrio* species in a number of Northwest European countries, in recent years). This concern also applies on a global scale to most countries where human and non-human illnesses associated with these bacteria are increasing. However, until now there has been no observational or experimental evidence to support this view (as also applies more generally to the impact of climate change on the marine prokaryotic populations); this is mainly due to a lack of historical data. Answering this global concern is pivotal to understanding, predicting and potentially managing climate change impacts on human and animal health associated with our oceans.

2 Methods

The Continuous Plankton Recorder (CPR) survey is one of the longest running marine biological monitoring programmes in the world, and provides a long-term archive of formalin preserved plankton samples (http://www.sahfos.ac.uk). We exploit the well association between vibrios and plankton, that is considered to be one of the largest reservoir of these bacteria in nature, to assess a possible linkage between *Vibrio* occurrence in the sea and environmental variables (sea surface temperature, phytoplankton and zooplankton abundance) over a decadal scale by applying a molecular analysis to the microbial community on historical CPR samples.

To this end we developed an unbiased index of abundance for *Vibrio* quantification in CPR samples, termed a ‘*Vibrio* relative abundance index-VAI’ (Figure 1).
This index measures the relative proportion of the plankton associated bacteria that are vibrios and is defined as the ratio of *Vibrio* spp. cells to the total number of bacterial cells assessed by real-time PCR using genus-specific and universal primers, respectively. About 100-bp amplicons were selected, allowing amplification to take place and maximising the reaction yields when the DNA was fragmented. In addition, PCR protocols based on similar-size amplicons (113 vs. 98 bp for *Vibrio* and total bacteria, respectively) were used assuming that DNA damage over time, including fragmentation, was the same for both amplified fragments. Using this approach we were able to measure and compare relative *Vibrio* abundances in CPR samples from different years. To assess *Vibrio* dominance within the whole bacterial community, 16S rDNA pyrosequencing analysis was also carried out on selected CPR samples.

### 3 Summary of Results

Potential future increases in marine vibrios and their associated diseases as a consequence of ocean warming is an emerging global concern. In this study, we were able to recover environmental DNA from CPR samples that had been stored for up to ~50 years in a formalin-fixed format, which is suitable for molecular analyses of the associated prokaryotic community. For the first time we show, by retrospective molecular analysis of plankton samples that vibrios, including the species *V. cholerae*, have increased in prevalence in the last 50 years in the coastal North Sea and that this increase is correlated significantly with increasing sea surface temperature during the same period (Figure 2).
In addition, using pyrosequencing, we provide evidence that bacteria belonging to the genus *Vibrio* not only increased in relative abundance over the last half century in the southern North Sea, but also became dominant within the plankton-associated bacterial community of coastal marine waters.

Our findings provide support for the view that global warming is having a strong impact on the composition of marine prokaryotic communities with potential important implications for human and animal health. This study also provide a proof of concept for the assessment of microbial diversity from the large and ocean basin-wide collection of formalin preserved marine biological samples obtained by the CPR survey opening up a novel window for the long-term and retrospective study of microbial biodiversity and the global ecology of marine bacterial communities.
Literature


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A survey of *Vibrio parahaemolyticus* in French Atlantic coastal waters

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1 Introduction

*Vibrio parahaemolyticus* is part of the natural flora of estuarine and coastal marine environments worldwide and it has been isolated from water, sediments and a variety of shellfish species from Europe (Hervio-Heath et al. 2002, Roque et al. 2009, Deter et al. 2010, Gugliandolo et al. 2011). There has been an increase in the incidence of foodborne *Vibrio* disease and the health risk associated with enteropathogenic *V. parahaemolyticus* is a major concern. Because of this inherent risk, it is relevant to understand the mechanisms that affect the natural population of *V. parahaemolyticus* in the environment. Numerous studies in coastal and estuarine areas have been performed to identify the causative or associative factors that affect *V. parahaemolyticus* incidence (Martinez-Urtaza et al. 2008) but few data were reported for enteropathogenic *V. parahaemolyticus*.

Specific objectives of this study were 1) to evaluate the occurrence of this bacterium in seawater, shellfish and superficial sediments from three shellfish growing sites in Pertuis Breton, 2) to understand the interaction of the phytoplankton population composition with the population dynamics of total and enteropathogenic *V. parahaemolyticus*, and 3) to model the occurrence of *V. parahaemolyticus* as a function of major environmental parameters in order to understand how changing climate conditions might affect the ecology of *V. parahaemolyticus* in the environment. The goal is to predict the likelihood of the presence of *V. parahaemolyticus* in these compartments by a multivariate empirical habitat model.

2 Materials and methods

2.1 Detection of *V. parahaemolyticus* and environmental variables

In this study, the occurrence of total and enteropathogenic *V. parahaemolyticus* was investigated with respect to temperature, salinity, turbidity, chlorophyll *a* and association with phytoplankton.

Seawater and mussel (*Mytilus edulis*) samples were collected monthly at three sites located in a shellfish growing area (Pertuis Breton, Atlantic coast) between April 2008 and March 2011. Two of the sites, Eperon and La Carrelère, were coastal and close to freshwater inputs from Le Lay and La Sèvre, respectively. The third site, Filière W, was located at approximately
7 kms from the coast and away from the two rivers (Figure 1). Superficial sediments were taken from two of the sites, La Carrelère and L’Eperon. Water temperature and salinity were recorded at the time of sampling and seawater was later analyzed for turbidity, chlorophyll \( \alpha \) and phytoplankton species and abundance.

![Map of the study area (Pertuis Breton, Atlantic coast, France). Seawater and mussels were sampled at Filière W, Eperon and La Carrelère. Superficial sediments were collected at Eperon and La Carrelère.](image)

Figure 1: Map of the study area (Pertuis Breton, Atlantic coast, France). Seawater and mussels were sampled at Filière W, Eperon and La Carrelère. Superficial sediments were collected at Eperon and La Carrelère.

Enrichment in Alkaline Peptone Water (APW) coupled with real-time PCR was used to detect and confirm the identification of \( V. \) parahaemolyticus. Enrichments were spread onto TCBS agar and CHROMagar\textsuperscript{TM} Vibrio and presumptive \( V. \) parahaemolyticus colonies were isolated and purified on Heart Infusion Agar (HIA 2 % NaCl). DNA was extracted from the enrichments and presumptive \( V. \) parahaemolyticus colonies. Real-time PCR was performed targeting the \( toxR \) gene for identification at the species level (total \( V. \) parahaemolyticus). Enrichments and colonies positive for \( V. \) parahaemolyticus were subjected to further analysis for hemolysin genes, \( tdh \), and \( trh1,2 \) for the detection of enteropathogenic \( V. \) parahaemolyticus.

2.2 Data analysis

\textit{Vibrio parahaemolyticus} Empirical Habitat Suitability Model

Because the methods used for detection of \( V. \) parahaemolyticus were not quantitative, a generalized linear model (GLM) with a binomial distribution, also called logistic regression, was used to model the presence-absence of total and enteropathogenic \( V. \) parahaemolyticus using R software. A stepwise selection process was used, in which descriptive variables (water temperature, turbidity, salinity and chlorophyll \( \alpha \)) were entered in turn into the model. Variables remained in the model only if they were significant (\( \alpha<5 \% \)) at each step, when a new variable was added.
Phytoplankton Community Composition

A Monte Carlo approach was used to detect significant association between occurrence of \textit{V. parahaemolyticus} in one of the three compartments (i.e., mussels, water, and superficial sediment) and presence of a specific phytoplankton species. The number of simultaneous occurrences of positive observations in the two variables was divided by the number of observations. The bootstrap procedure, employing 100,000 series generated randomly and preserving the same first-order autocorrelation structure, as observed in the data, was established to compute a stable distribution of the statistic that can be expected to occur randomly. The P-value of the test corresponds to 1 minus the rank of the observed statistic over the 100,000 statistics obtained randomly and divided by 100,001. If the P-value was equal to or less than a significance level of 5\%, we considered the association to be significantly different from an occurrence by chance. There is a mechanistic basis expecting a lag of up to one month from occurrence of phytoplankton and subsequent presence of \textit{V. parahaemolyticus} in the environment.

3 Results and discussion

3.1 \textit{V. parahaemolyticus} in seawater, mussels and superficial sediments

\textit{Vibrio parahaemolyticus} was found in 50 (42.7\%), 54 (46.2\%) and 39 (60\%) of 117 seawater, 117 mussel and 65 superficial sediments samples analyzed, respectively. The genes \textit{trh1, 2} and thus, enteropathogenic \textit{V. parahaemolyticus} were detected in 22 (18.8\%), 26 (22.2\%) and 32 (49.2\%) samples of seawater, mussels and superficial sediments, respectively. In contrast, \textit{tdh}-positive \textit{V. parahaemolyticus} were found in 3 mussel samples only, one in Filière W (summer 2008) and two in La Carrelère (twice in winter 2010 - 2011). Total and enteropathogenic \textit{V. parahaemolyticus} showed a seasonal pattern (detection during the late spring, summer and autumn months) in seawater and mussels. These bacteria occurred all year round in the superficial sediments of La Carrelère and Eperon.

Significant differences in the presence of total and enteropathogenic (\textit{trh1, 2}) \textit{V. parahaemolyticus} were observed between the two coastal sites and Filières W. However, no significant differences were found between the 3 sites in terms of seasonality.

3.2 Environmental factors

Seawater temperatures were highly similar and showed a seasonal pattern at the three sampling sites with values varying from 6.0 °C in winter to 21.0 - 23.0 °C in summer. In contrast, salinity showed a high range of variations with values fluctuating from 24 to 35 ppt, 12 to 35 ppt and 14 to 35 ppt, in Filière W, Eperon and la Carrelère, respectively. The decrease in and the lowest values of salinity at Eperon and La Carrelère were recorded from December to June and were arising from heavy rainfalls and freshwater inputs from the rivers. Turbidity and chlorophyll \textit{a} showed narrower ranges of variations except for Eperon where values of turbidity varied from 2.7 to 61.2 NTU and of chlorophyll \textit{a} from 0.24 to 11.92 mg/m\textsuperscript{3}. 

3.3 Effect of environmental variables on the presence of total and enteropathogenic *V. parahaemolyticus*

In the three sites, seawater temperature was the only environmental variable that significantly affected the presence of total *V. parahaemolyticus* in seawater and mussels although it explained only from 13.8 to 36 %, 33.7 to 36.2 % and 39.4 % of total deviance in mussels from the three sites, seawater from Eperon and La Carrelère and sediment from Eperon, respectively, suggesting that additional environment variables may play a role as well.

The presence of enteropathogenic *V. parahaemolyticus* was explained only in the mussels from Eperon. In this case, a significant effect of chlorophyll a, salinity and of combined chlorophyll a and salinity accounted for 68.2 % of the total deviance.

3.4 Phytoplankton population composition

Among the 51 species of phytoplankton identified at Eperon, the occurrence of 10, and 5 species belonging to Diatoms were significantly associated to the presence of *V. parahaemolyticus* without lag time and with one month lag time, respectively. Among them, four were significantly associated to the occurrence of *V. parahaemolyticus* without and with one month lag time.

4 Conclusions

Overall, total *V. parahaemolyticus* were present in seawater and mussels from the three sites and in sediment from Eperon and La Carrelère. Enteropathogenic *V. parahaemolyticus* (*trh1, 2*-positive) were detected in all compartments with prevalence of 18.8 %, 22.2 and 49.2 % in seawater, mussels and sediment, respectively. These prevalence are similar to those reported for seawater and mussels in the one-year study performed on these three sites (DETER et al. 2010).

Using generalized linear models we showed that seawater temperature was the main factor affecting significantly the occurrence of total *V. parahemolyticus* in seawater, mussels and sediment from the shellfish growing area selected for this study. This is in agreement with previous studies reported in Europe (VEZULLI et al. 2009). In the case of enteropathogenic *V. parahaemolyticus*, the results obtained for shellfish support that environmental variables such as salinity, chlorophyll a and interaction with phytoplankton should be investigated further to make robust models predicting the presence of potentially pathogenic *V. parahaemolyticus* in temperate waters.

Literature


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Pathogenic vibrios in marine and fresh recreational waters in The Netherlands related to environmental conditions and health complaints

Ciska Schets, Harold van den Berg and Ana Maria de Roda Husman

1 Introduction

During the warm summer of 2006, an increase in the number of bathers with *Vibrio* infections was reported from several countries in North-Western Europe; e. g. wound infections associated with contact with Baltic Sea water in Germany (FRANK et al. 2006) and Sweden (ANDERSSON & EKDAHL 2006). In the Netherlands, *V. alginolyticus* caused wound infections in two persons and ear infections in another two persons after swimming in the Oosterschelde, a large inlet on the North Sea (SCHETS et al. 2006, 2011).

In the European Union, bathing waters are monitored for faecal indicator parameters according to European Bathing Water Directive 2006/7/EC (2006) which does not address the monitoring of indigenous waterborne pathogens such as *Vibrio* spp.

This study aims at the detection and quantification of potentially human pathogenic *Vibrio* species in recreational waters in the Netherlands during a five-year period, starting in 2009.

2 Materials and methods

Six recreational waters were sampled during the summers of 2009, 2010 and 2011. All samples were pre-enriched in alkaline buffered peptone water (ABPW) incubated at 41.5 ± 1 °C for 18 - 20 h; additionally, variants of this enrichment procedure were tested. ABPW enrichments were cultured on Trypton Citrate Bile Sucrose (TCBS) agar incubated at 36 ± 2 °C for 16 - 20 h. The 2011 ABPW enrichments were additionally cultured on chromID Vibrio agar (VID). For each sample, a maximum of ten presumptive *Vibrio* spp. colonies per enrichment or culture medium was obtained in pure culture and further tested and identified as described by SCHETS et al. (2010). *Escherichia coli* and enterococci were enumerated by using membrane filtration methods according to ISO 9308-1 (Rapid Test) (2000) and ISO 7899-2 (2000), respectively. The maximum likelihood method was used to estimate *Vibrio* MPN in water samples by using Mathematica 5.1.

With the help of clinical microbiology laboratories and general practitioners, surveillance for patients with recreational water related ear and wound infections caused by *Vibrio* spp. was performed.
3 Results

Pathogenic *Vibrio* species were detected in all studied recreational waters albeit in variable numbers. Although a higher proportion of the samples were positive at elevated water temperatures, a clear quantitative relation between *Vibrio* numbers in water samples and the water temperature was not observed. This may be explained by maximum water temperatures of around 21 °C during the summers of 2009, 2010 and 2011.

A total of 1606 presumptive *Vibrio* strains were isolated; the most frequently isolated species was *V. alginolyticus* (52 %), followed by *V. cholerae* non-O1/O139 (15 %), *V. parahaemolyticus* (11 %) and *V. fluvialis* (2.1 %). Of the presumptive *Vibrio* isolates, 18 % were non-*Vibrio*, but were not further identified. Different *Vibrio* species were isolated from the different sites, reflecting their ability to tolerate different salinities (Figure 1).

![Figure 1](image)

*Figure 1:* *Vibrio* species isolated from various recreational water sites in the Netherlands

A combination of sample pre-enrichment in ABPW incubated at 36 ± 2 °C in ABPW incubated at 41.5 ± 1 °C, both for 18 - 20 h, yielded the highest number of positive samples and the broadest range of isolated *Vibrio* species. Both on TCBS and on VID agar, *Vibrio* species can be distinguished by their colony colour, however, due to gradient colony colours on both agars, confirmation is necessary. It was observed that over 25 % of characteristic colonies on VID were non-*Vibrio*, compared to approximately 10 % of the characteristic colonies on TCBS.

Active surveillance yielded one patient with a recreational water related *V. cholera* non-O1/O139 wound infection in 2009 and one patient with an otitis externa caused by *V. alginolyticus* in 2010. From one patient with a wound infection and another patient with an ear infection, both after swimming in the Oosterschelde in 2011, no *Vibrio* spp. could be isolated.
4 Conclusions

> Human pathogenic *Vibrio* spp. have been isolated from Dutch recreational waters and mainly included *V. alginolyticus*, *V. parahaemolyticus* and *V. cholerae* non-O1/O139.

> Detection of the presence of total *Vibrio* spp. in water samples and isolation of the species present is optimal when a combination of pre-enrichment in ABPW incubated at 41.5 ± 1 °C and at 36 ± 2 °C, for 18 - 20 h is applied, followed by cultivation on TCBS.

> The number of patients that annually contracts a *Vibrio* infection through recreational water exposure in the Netherlands seems low, but may be underestimated.

Summary

Several marine *Vibrio* species are human pathogens that have increasingly been associated with wound and ear infections after exposure to contaminated bathing waters in North-West Europe. Human pathogenic *Vibrio* species were isolated from six official bathing sites in The Netherlands. Isolated *Vibrio* species included *V. alginolyticus*, *V. parahaemolyticus*, *V. cholerae* non-O1/O139 and *V. fluvialis*. Although more samples were positive at elevated water temperatures, a clear quantitative relation between *Vibrio* numbers in water samples and the water temperature was not observed which may be explained by low water temperatures. Active surveillance yielded a limited number of human recreational water related *Vibrio* infections.

Literature


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Vibrio spp. in Norwegian blue mussels and seawater

Anette Bauer Ellingsen and Liv Marit Rørvik

1 Introduction

Vibrio spp. in Norwegian seafood and seawater have not been of concern because of the normally low temperatures in the seawater. Vibrio cholerae is the only notifiable species, 11 cases were registered from 1985 till today. The cases are normally contracted abroad, though two cases were not linked to travel or imported food. Vibrio parahaemolyticus is sporadically isolated from patients, but last year the bacteria were isolated from a cluster of cases in the south west part of Norway. The possible outbreak has not been solved. Vibrio vulnificus has been isolated from wound infections. Little was known of the occurrence of Vibrio spp. in Norwegian seawater and seafood until the Food Safety Authorities funded a couple of projects on the subject from 2001 to 2006.

2 Materials and methods

Samples of blue mussels were collected from the whole coast over the whole year, with emphasis on the southern part of the country, during the summer months. Seawater samples were collected from the southeast coast, mainly the Oslo fjord during summer.

2.1 Isolation and identification of Vibrio spp.

The samples were analysed according to NMKL No. 156, a conventional cultivation method. The isolates were identified biochemically, as well as with PCR targeting ToxR genes for the three species.

2.2 Virulence gene analysis

Isolates of V. parahaemolyticus were analysed for tdh and trh, and V. cholerae for ctx by PCR. Several isolates from each positive sample were examined for virulence genes.

2.3 PFGE, MLST

Clinical isolates, kindly provided by National Institute of Public health, all trh+ isolates from the surveys, and a, selection of isolates of V. parahaemolyticus, representing the whole coast, as well as water isolates, were typed by PFGE, according to MARTINEZ-URTAZA et al. (2004), using NotI. MLST was performed on trh+ isolates according to GONZÁLEZ-ESCALONA et al. (2008).
3 Results

The occurrence of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* in five surveys from 2001 to 2006 is shown in Table 1.

Table 1

<table>
<thead>
<tr>
<th>Sample type</th>
<th>n</th>
<th><em>V. parahaemolyticus</em> positive samples</th>
<th><em>V. cholerae</em> positive samples</th>
<th><em>V. vulnificus</em> positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue mussels</td>
<td>1003</td>
<td>137 (14 %)</td>
<td>13 (1 %)</td>
<td>4 (&lt;1 %)</td>
</tr>
<tr>
<td>Crab</td>
<td>14</td>
<td>1 (2 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oyster</td>
<td>16</td>
<td>1 (11 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sea water</td>
<td>131</td>
<td>89 (68 %)</td>
<td>6 (5 %)</td>
<td>7 (5 %)</td>
</tr>
</tbody>
</table>

Blue mussels from four sampling areas at the southeast coast represented 18 % of the samples, but accounted for 73 % of the positive samples. *V. parahemolyticus* from five blue mussel and four seawater samples harboured *trh, tdh* was not detected. No *V. cholerae* isolates harboured *ctx*. The *trh*+ *V. parahaemolyticus* were isolated from the south coast.

Diversity among *V. parahaemolyticus* isolates as shown by PFGE results was great. The isolates (n=236) could be divided into 103 pulsotypes, and 72 clusters. The *trh*+ isolates belonged to two clusters, none of them included *trh*- isolates. Certain pulsotypes, including *trh* + variants, seemed to persist at distant locations. MLST results supported the PFGE findings, revealing that some pathogenic (*trh+*) and non-pathogenic *V. parahaemolyticus* are highly genetically related.

4 Discussion

Results from surveys on blue mussels and seawater for pathogenic *Vibrio* spp. in 2001 - 2006 show that *V. parahaemolyticus* occurred in the Norwegian marin environment to some extent, while *V. cholerae* and *V. vulnificus* were sporadically isolated. The *Vibrio* spp. were mainly isolated during the summer months, and from the south coast. This could be expected, as the water temperature on the west and north coast is rather low the whole year. Most of the environmental isolates did not harbour the virulence genes *tdh or trh*, but the detection of some *trh*+ isolates showed that there is a potential for human infection from Norwegian blue mussels.

No surveys have been performed on pathogenic *Vibrio* spp. in the Norwegian environment or mussels since 2006. Taken into account the cluster of *V. parahaemolyticus* cases in 2011, as well as the climate change threat, these bacteria should be monitored on a more regular basis.
Literature


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2002 - 2008: Pathogenic Vibrio spp. in Norwegian blue mussels and seawater.

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Geothermal locations at the Icelandic coast as a habitat for *Vibrio cholerae*

Eva Benediktsdóttir and Herdís Eva Hermundardóttir

1 Introduction

As many as 52 natural hot springs have been reported along the coastline of Iceland (BENJAMINSSON 1988), thereof 37 geothermally active sites that come up at low tide but mix with the sea between. The water has been measured above 30 °C in 31 sites. Most of these are situated along the south-west and northern coast of Iceland, in the low-temperature geothermal fields. Measurements have revealed that the water is most often coming from deep fresh groundwater, alkaline and low in sulfide (BENJAMINSSON 1981). Moreover, many villages in the northern and western parts of the island are heated with geothermal fresh water, and overflow from such operations runs into the coast. A rapid mixing with seawater occurs outside the outlets, where the decline in temperature is dependent on the quantity and temperature of the geothermal water, the diurnal tidal changes and to the surge and weather conditions, resulting in locations characterized by fluctuating temperature and salinity and surrounded with sea water below 10 °C for nine months of the year and up to 12 - 14 °C in July.

Mesophilic *Vibrio* bacteria are mainly found in the marine environment in temperate, semi-tropical, or tropical regions, and their abundance is associated with high sea surface temperature and the level of organic material in the sea or sediment. Many of those are pathogenic for humans. In a study that was carried out on vibrios off the coast around Reykjavík in the early nineties, mesophilic vibrios were not found. In 2006, a survey was started to investigate the presence of mesophilic vibrios in geothermally influenced sea water, with the result that *V. cholerae* was isolated from all such locations sampled, but samples from locations that were not influenced by hot water were negative. A total of 127 of the isolated *V. cholerae* strains have been tested for different virulence genes using PCR, and all tested negative for the cholera toxin subunit genes and toxin co-regulated pilus, TcpA, but positive for *toxR*, *ompU*, *hlyA*, *rtxA*, HA/P and *luxO*. 124 strains were positive for *nanH*, that codes for sialidase, and 58 strains contained *chxA*, that encodes cholix toxin. PFGE, on a limited number of strains isolated from different locations in Iceland, showed genomic variability within the Icelandic *V. cholerae* populations, and phenotypic diversity was also shown using Biolog Phenotype MicroArrays™ (HALEY et al. 2012). A survey is going on to study the environmental conditions and the biotic reservoirs of *V. cholerae* in the thermally influenced locations, as well as the dispersal of *V. cholerae* from these locations.
2 Materials and methods

Four sites have been selected as representatives of different habitats and locations:

a) Ægissíða, Reykjavík: An overflow outlet from the heating utility in Reykjavík, a few liters/sec (variable) of warm geothermal water, 30 - 35 °C, runs into the intertidal zone. The pipe is covered at high tide by sea water. The coast is rich in macroalgae.

b) Berserkseyri, Snaefellsnes peninsula in a rural area at the west coast of Iceland: A mixture of a sand beach and lava covered by macroalgae vegetation, southern coast of Breiðafjörður. The spring, 78 °C and with estimated flow a few l/sec, is situated ca. 1 m above the tidemark.

c) Skarðshver, Vatnsnes, situated in a rural area at the northern coast of Iceland: A natural hot spring, 70 - 80 °C, emerges at low tide, but is covered at high tide. The coast is rich in vegetation.

d) At Reykjanesvirkjun below a geothermal power station in a rural area at the southwest coast: Hot underground seawater, with full salinity, 60 °C, is pumped up at magnitude 4000 l/sec, and runs into the sea in two pipes about one m above the sea level at high tide. Large rocks and lava are dominant in the intertidal zone with no sand, and some precipitation is seen around the flow. The site is open for the ocean and marked by heavy waves. The hot water influences the temperature around the outlet also at high tide.

All samples were taken at low tide. One liter sea samples were filtered through 0.2 μm pore size membrane filters that were incubated in APW. Macro-organisms were washed three times with sterile NaPBS solution and homogenized before incubation. Sediment samples were washed with sterile 200 ml NaPBS and macro-organisms separated by filtering through a sieve with 90 μm pore size. All samples were incubated in APW at 37 °C followed by cultivation on TCBS agar (HUQ et al. 2006). Green and yellow colonies were isolated on TSB agar and analyzed by biochemical tests and PCR using *V. cholerae* specific primers targeting the 16s to 23s intergenic spacer region. DNA was extracted as well from 1 ml of APW after 24 and 48 h cultivation and tested for *V. cholerae* by PCR. Two or three sea samples were taken on each sampling occasion.

3 Results and discussion

Water samples have been collected at Ægissíða 10 times during five winters, from September to March, and samples have tested positive for *V. cholerae* on all occasions with only two samples negative. Samples have been taken 1 km away from the outlet five times, resulting in one sample positive for cultivable *V. cholerae* and four negative. The sea at Berserkseyri has been sampled three times below the spring and once 2 km away, and all have tested positive for *V. cholerae*. At Skarðshver, water samples have been collected three times, and once 400 m, 2 km and 10 km away from the spring, and all except the sample taken 10 km away from the spring tested positive for *V. cholerae*. At Reykjanesvirkjun sea samples have been collected four times near the outlet and once 4 km away. No sea sample from Reykjanesvirkjun have tested positive for *V. cholerae*. 
Samples have been taken from macroalgae, sediment and macroscopic animals at all sites if present. Samples that were collected at Reykjanesvirkjun were negative for \textit{V. cholerae}, but positive results were obtained at the three other sampling sites influenced by the hot water. Macroscopic organisms positive for \textit{V. cholerae} hitherto are macroalgae, burrowing lug-worm, amphipods, rough periwinkle and mussels. 61\% of sediment samples collected at 4 - 17 °C have been positive.

\textit{V. cholerae} is known to be involved in surface biofilm formation, and it is associated with diverse organisms including zooplankton, phytoplankton, oysters, crabs, fish and chironomid egg masses. (reviewed by VEZZULLI et al. 2010). The geothermal water from the hot springs and outlets at the coast is constantly surged into the surrounding water with temperatures below the growth temperature of \textit{V. cholerae} most part of the year. This fact makes it implausible that the biotic reservoir at the Icelandic coast is planktonic, which is supported by our results that show that \textit{V. cholerae} is readily isolated from the benthic fauna and flora. \textit{V. cholerae} is able to reside and grow at temperatures fluctuating far below their minimum growth temperature every day, and they are dispersed in the sea from the thermally influenced locations. Former results have indicated that the \textit{V. cholerae} species is native at the Icelandic coast and not imported or derived from a single source (HALEY et al. 2012). The negative results obtained in samples taken at Reykjanesvirkjun are puzzling, but the full salinity of the water and the harsh environment with a poor fauna and flora are a plausible cause. Further studies on the dispersal of vegetative as well as VBNC \textit{V. cholerae} cells from the geothermal locations along the coast of Iceland are in progress.

\textbf{Literature}


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The German zoonosis research network VibrioNet: First findings

Gunnar Gerdts, Thomas Alter, Edda Bartelt, Christina Frank, Florian Gunzer, Boris Oberheitmann and Eckhard Strauch

Bacteria of the genus *Vibrio* occur mainly in estuaries, marine coastal waters and sediments, free living or attached to particles. Several *Vibrio* species are serious human pathogens. Contact with contaminated water and consumption of raw seafood are the main infection factors for *Vibrio* associated diseases. Traditionally, most gastroenteritis outbreaks caused by *V. parahaemolyticus* are linked to warmer regions but an increase of pathogenic *V. parahaemolyticus* also in temperate waters can be expected. Global warming leads to rising water temperatures and therefore expanded niches for mesophilic *Vibrio* spp. Climate change could therefore have direct influence on the growth of *Vibrio* spp. and might also contribute to a more northerly geographic dispersal of pathogenic *Vibrio* spp.

To this date, studies about potentially human pathogenic *Vibrio* spp. such as *V. parahaemolyticus* or *V. vulnificus* are still rare in Northern Europe. In order to improve our understanding of climate-driven changes of incidences of vibrioses and to assess the risk for the German population, it requires (i) a systematic surveillance programme for non-cholera *Vibrio* illnesses, (ii) standardized, validated, fit-for-purpose methodologies, (iii) experimental data on the molecular determinants of host-specific pathogenicity and transmission, and (iv) environmental data on the population dynamics and virulence characteristics of these pathogens. VibrioNet will jointly address these issues by bringing together expert researchers of marine ecology, infectious diseases, veterinary and human medicine, as well as governmental institutions and the food industry. Central to the research network will be the interaction with scientists from Asian and South American countries with a high incidence of *Vibrio* infections. After one year of intense research, we here present our first findings and results of the individual subprojects, collaborating in VibrioNet.

**Subproject C2: Seasonality of pathogenic *Vibrio* spp. in seawater, plankton, and shellfish of North and Baltic Sea (Alfred-Wegener-Institute for Polar and Marine Research)**

Subproject C2 aims to analyse potentially pathogenic *Vibrio* populations in German coastal waters and to describe the environmental parameters shaping the *Vibrio* community. In this context, a proper identification of environmental isolates is essential. Here we apply a polyphasic analysis approach with a focus on the usage of the Biotype© MALDI-TOF system. Until now about 800 *Vibrio* strains (mostly *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*) from diverse sources (North- and Baltic Sea; coastal and marine waters; seawater, plankton, shellfish and sediments) were included in our strain collection and are currently analysed by
molecular methods. From more than 300 strains MALDI-TOF reference spectra were already obtained and added to the Biotyper© database. Currently all strains are subjected to rpoB sequencing for affiliating phylogenetic information to MALDI-TOF spectra. Based on all analyses we aim to analyse the biogeography of V. cholerae, V. parahaemolyticus, V. vulnificus in German Waters and to provide a toolkit for fast microbiology of environmental Vibrio spp.

Subproject C3: Qualitative and quantitative detection of Vibrio spp. in mussels (Mytilus edulis) of bivalve mollusc harvesting areas and in Pacific Oysters (Crassostrea gigas) in the Lower Saxony Wadden Sea region (Institute for Fish and Fishery Products, Lower Saxony Federal State Office of Consumer Protection and Food Safety (LAVES))

Bivalve molluscs are an important food source in the European trade market. The microbiological criteria for classification and monitoring of bivalve molluscs harvesting areas are given in Regulation (EC) No 854/2004. The control programmes for the bivalve mollusc harvesting areas located at the Lower Saxony Wadden Sea region have been set up by the competent authorities, and according to the European Regulations they are based on the MPN-level of E. coli per 100 g of flesh and intravalvulare liquid of bivalve mussels. The rate of uptake and removal of indicator bacteria (such as E. coli) by bivalve molluscs differs from that of many pathogens that may be present, especially viral pathogens or pathogens of marine or estuarine environment, and therefore numbers of E. coli will not give an indication of the general risk of contamination by the pathogens. There is no correlation between the occurrence or numbers of Vibrio spp. and the faecal human pathogens or presently used indicators in a control program for harvesting areas. The existing monitoring programmes are not suitable to assess the risks of Vibrio spp. in bivalve molluscs. The objective of the study is the qualitative and quantitative detection of Vibrio spp., especially pathogenic Vibrio (V.) parahaemolyticus and V. vulnificus strains, at primary production level in mussels (Mytilus edulis) of bivalve mollusc harvesting areas of the Lower Saxony Wadden Sea region. Furthermore, the study aims to detect and quantify V. parahaemolyticus and of V. vulnificus strains in samples of Pacific Oyster (Crassostrea gigas) in the Wadden Sea, especially which are located nearby to mussel harvesting areas. Isolated strains of V. parahaemolyticus and of V. vulnificus of mussels and oysters will be stored in a strain collection and are intended for further determination of their cyto- and enterotoxigenic potential in cooperating laboratories.

At present, 176 samples of bivalve molluscs originating from harvesting areas and retail have been investigated. Blue mussels and Pacific oysters originating from the Wadden Sea during utmost one harvesting period showed a very high prevalence of Vibrio spp. (73.2 %, and 73.8 %, resp.), and each positive sample showed a contamination with at least two different Vibrio species. V. alginolyticus and V. parahaemolyticus have been detected in nearly each positive sample, whereas V. vulnificus, V. cholerae non O1/non O129 and V. fluvialis have been isolated only in a small number of samples. Only 22.7 % of sampled bivalve molluscs revealed a quantitative detection of Vibrio spp. using the MPN-method. The MPN load of V. parahaemolyticus varies between 3.6 and 1100 MPN/g flesh and intravalvulare liquid.

The investigations on their cyto- and enterotoxigenic potential are going on. Further investigations are planned to assess the risk of Vibrio contamination of bivalve molluscs with respect to the different harvesting periods and harvesting areas and to the comparison with packed bivalve mollusc products at borderline stations and at retail.
Subproject C4: Detection and proliferation of pathogenic *Vibrio* spp. in retail seafood

Subproject C-6B: Transcriptome profiling of pathogenic *Vibrio* spp. by microarray technique (Institute of Food Hygiene, Free University of Berlin)

Prevalence studies on *Vibrio* in mussels and shrimps in Germany on pre-harvest and retail levels showed high contamination rates with these bacteria so far. As far as food safety is concerned *Vibrio parahaemolyticus*, *V. vulnificus* and *V. cholerae* are the most important species within this genus. *Mytilus edulis* is one of the most frequently consumed mussels in Germany. Along with the water filtered by mussels, microorganisms such as *V. parahaemolyticus* and *V. cholerae* are accumulated. If these mussels are not heated thoroughly before consumption such bacteria could cause food borne infections. Because of the high prevalence of *V. parahaemolyticus* in seafood and its importance in public health, *Vibrio*-contamination and storage experiments were performed in *Mytilus edulis*. The aim of that study was to determine the clearance rate of *Mytilus edulis* after artificial *V. cholerae*, *V. parahaemolyticus* or *V. vulnificus* contamination. Moreover the kinetics of *Vibrio* loads in contaminated mussels under two different packings during storage at 3 °C or 15 °C were investigated. A fast accumulation of *Vibrio* spp. occurs in blue mussels within less than 2 h. Depuration over seven days reduces the bacterial load. However, depuration was not able to eradicate *Vibrio* spp. completely. Within 10 days of storage almost no reduction of the *Vibrio* load occurred in *Mytilus edulis*, except for variations in a 2 log10 interval. Additional to these investigations a genotyping by MLST (Multi-locus-sequence-typing) of selected strains isolated from shrimps originating from retail markets in Germany (shrimps originated from different Asian countries) and shrimp farms in Sri Lanka was performed. MLST analysis is a highly discriminating typing system on a nucleotide sequence based approach. The analyses of the strains showed a wide spread of Sequence Types (ST) all over Asia including 26 new ST. So far neither a country or region specific ST, nor a matrix specificity was found. This wide variety of ST within shrimps suggests the uptake of environmental strains to be dominant. More *Vibrio* strains from other regions need to be tested.

In order to investigate survival mechanisms at lower temperatures and the impact of temperature on expression of virulence genes, whole genome microarray analysis was carried to compare gene expression at different temperatures (4 °C, 15 °C, 20 °C and 37°C). First data show that lowered temperatures result in a large set of differentially expressed genes that can be clustered into groups. Currently the gene content of these groups is investigated.

Subproject C5: Toxigenic potential of *Vibrio* spp. from environmental, food, and clinical sources in Germany

Subproject C-6A: Molecular adaptations of pathogenic *Vibrio* spp. to bivalve and mammalian hosts: Interactions of pathogenic vibrios with eucaryotic cells (Federal Institute for Risk Assessment)

In individual project C-5 and C-6A phenotypic and genotypic traits of sets of isolates of *V. parahaemolyticus* and *V. vulnificus* from different sources are comparatively evaluated to assess their pathogenic potential. Molecular typing using Multi-Locus Sequence Typing and virulence markers are used to evaluate the genetic profiles and virulence potential. Phenotypic virulence characteristics are addressed using tests for pathogenicity related traits such as serum resistance, hemolysin production, motility, biofilm formation and extracellular enzyme activities. Finally, selected strains are characterized in in vitro screens using cell cultures.
First results of these investigations indicate that there are great variations concerning these phenotypic traits in both species. Remarkably, serum resistance is highly variable. While some stains are completely resistant to media containing 80% human serum others are inhibited at low concentrations (<10% serum content). In *V. vulnificus* serum resistance has been attributed to a genetic polymorphism in the *pilF* gene and currently we are sequencing the corresponding genes of all investigated strains from the Baltic Sea region. In another approach the 16S rRNA types of *V. vulnificus* strains are investigated. Two rRNA types have been described (A- and B-type) with most clinical strains belonging to the B-type.

**Subproject C 7: Pilot studies on the human burden of wound and diarrhoeal *Vibrio* spp. infections in Germany (Robert-Koch-Institute)**

The subproject of the Robert Koch Institute pertains the epidemiology of human non-cholera *Vibrio* infections in Germany. Though cases of wound and gastroenteric infections with non-cholera *Vibrio* have been described in Germany, the incidence of these infections is not clear as the pathogens are not required to be notified according to the German Protection against Infection Act (IfSG). However, as such infections can be notified in the category of “other threatening diseases”, one intention of the subproject is to appeal for voluntary notification and to record new infections over the next years in the national data base. The other intention is to analyse retrospective data of clinical and laboratory records of the past ten years for possible non-notified or non-cholera *Vibrio* infections not diagnosed as such. These data will be analysed for a potential link between high sea surface temperatures (in the context of climate change) and incidence of possible or confirmed non-cholera *Vibrio* infections. Cooperating with the subproject of the technical university of Dresden, any gastroenteric non-cholera *Vibrio* infections identified in their active case search will be included in a case-control-study looking for risk factors for gastroenteric infections with non-cholera *Vibrio* in Germany.

**Subproject C8: Incidence of *Vibrio* spp. isolates in human stool specimens and evaluation of their virulence potential in in vivo model systems**

**Subproject C-6C: Molecular characterization of pathogenic markers in *Vibrio* by whole genome sequencing**

(Institute for Medical Microbiology and Hygiene, TU Dresden)

The two subprojects are focused at estimating the incidence of Non-Cholerae *Vibrio* spp. in human stool specimens followed by evaluation of their virulence potential and the complete genome sequencing and annotation of interesting *Vibrio* strains isolated by the consortium. Currently, stool samples from the Dresden University Hospital and from the Dresden outpatient’s clinic are tested for presence of Non-Cholerae *Vibrio* spp. A first edition of a *Vibrio* pathotyping array has been manufactured which is being evaluated with sequenced *Vibrio* spp. type strains. Furthermore, whole genome sequencing of five *V. vulnificus* and one *V. alginolyticus* patient isolates, as well as two additional *Vibrio* strains provided by two other subprojects, is underway.
Subproject C9: Development of rapid, accurate and affordable PCR-based detection systems for pathogenic *Vibrio* spp. (Q-Bioanalytic GmbH)

One goal of the subproject is the development and validation of Real-Time PCR test kits for the *Vibrio* species *cholerae*, *parahaemolyticus*, *vulnificus* and *alginolyticus* including the detection of relevant toxic variants. The application of the these rapid assays in food safety and environmental testing can improve consumer’s safety and medical diagnostics and can help to standardize environmental surveillance programmes for *Vibrio* species in coastal regions. Real-Time PCR test kits for *V. cholerae* and *V. parahaemolyticus* were validated and an internal amplification control was included in the assay. The validation study included testing for inclusivity, exclusivity, limit of detection and testing in food matrices. For *V. cholerae* Real-Time PCR tests for the pathogenic strains carrying the *ctx* gene were also developed. The test kits for *V. vulnificus* and *V. alginolyticus*, were developed and are currently under detailed validation. The *V. cholerae* Real-Time PCR tests were successfully investigated with 20 target strains and 30 non target strains. The same was done with *V. parahaemolyticus*. The sensitivity testing revealed a limit of detection of below 100 CFU without pre-enrichment in pure culture and in matrix. Thus the limit of detection is more than sufficient for a test that includes a pre-enrichment step, where cell counts of more than 1 Mio. CFU are expected.

The test kits developed assure a rapid and sensitive detection of *Vibrio* species. Further studies for development of tests and their validation are on-going.
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Pathogenic *Vibrio* species in German coastal waters of the North Sea and the Baltic Sea – a comparison

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1 Introduction

*Vibrio* spp. are ubiquitous bacteria naturally found in marine and estuarine waters. Human pathogenic representatives of this genus can cause severe wound, ear and eye infections associated with bathing water contact. In the hot summer of 1994, first incidences of *Vibrio vulnificus*-related wound infections occurred among bathers at the Baltic Sea coast in Germany, Denmark and Sweden (HOYER et al. 1995, DALSGAARD et al. 1996, MELHUS & HOEKSTRA 1995). Since then sporadic cases of *Vibrio*-related infections – some of them lethal – have been repeatedly reported for the Baltic Sea (BS) and, less frequently, for the North Sea (NS) coast during summer heat waves. Not only average sea surface temperatures (SST) have increased considerably in the NS and the BS over the last decades, but also the probability of warm weather aberrances (MACKENZIE & SCHIEDEK 2007, WILTSHIRE & MANLY 2004). Recent studies show that the land-locked European Seas are particularly vulnerable towards climate warming (BELKIN 2009) and epidemiological data suggest a linkage between SST anomalies and *Vibrio* infections in these areas (BAKER-AUSTIN, unpublished data).

Although concern is growing that *Vibrio* infections may become an emerging disease in Northern European coastal waters, there is only insufficient knowledge of the ecology of vibrios and their health risk potential in these regions, however. Therefore, two independent monitoring studies were carried out at the German BS and NS coast, in cooperation with the local health agencies, the Governmental Institute of Public Health of Lower Saxony (NS) and the Governmental Institute of Public Health and Social Affairs of Mecklenburg-Western Pomerania (BS). At the NS, monthly water and sediment samples from 10 bathing sites were tested for potentially pathogenic *Vibrio* spp. over a 2-year period, starting in December 2009. In the BS, water and sediment samples from 12 sampling stations were analyzed in the course of 6 sampling campaigns between October 2010 and April 2012, including 2 bathing sites in the Western BS as well as 5 stations in the Darss-Zingster Bodden and 5 sites in the Greifswalder Bodden (Figure 1). A range of contextual data were collected in lock step. Aim of both studies was to investigate seasonal variations of *Vibrio* spp. in these waters, to identify environmental parameters that promote *Vibrio* proliferation and to identify possible “hot spots” of *Vibrio* spp. occurrence in German coastal waters. Preliminary results are presented at the symposium.
Figure 1: Location of the 12 sampling sites at the German Baltic Sea coast. 2 sites along the Mecklenburg-Western Pomeranian coast (Wohlenberger Wiek and Warnemünde Weststrand), 5 sites in the Greifswalder Bodden (GB 1 - GB 5) and 5 sites in the Darß-Zingster-Bodden (DZB 6 - DZB 10).

2 Measuring principles

2.1 Vibrio spp. analyses

All analyses were directed towards the detection of 4 potentially pathogenic Vibrio species: V. vulnificus, V. cholerae, V. alginolyticus and V. parahaemolyticus using different culturing approaches. For the detection of Vibrio spp. in water samples, water was either membrane-filtered or directly plated, using CHROMagar Vibrio (NS) or TCBS (BS) as selective culturing media. For the detection of Vibrio spp. in sediments, a direct and an indirect approach was chosen. The direct approach built up on the MPN technique and involved a fourfold dilution of the sediment samples in buffered peptone and overnight incubation at 36 °C, followed by sub-cultivation on CHROMagar Vibrio (NS) or TCBS (BS), respectively. For the indirect approach, one part of sediment was mixed with one part of aqua dest. and one part of synthetic sea salt solution for 30 min. on a magnetic stirrer in order to extract the bacteria from the sediments. After settlement of the sediment, the supernatant was removed and subjected to membrane filtration or direct plating using CHROMagar Vibrio (NS) or TCBS (BS) plates. Enumeration was based on colony morphology. In the case of the NS samples, presumptive V. vulnificus and V. cholerae were sub-cultured on TCBS for better differentiation. Colonies destined for further species identification were sub-cultivated on Columbia blood agar prior to API and serological testing (V. cholerae only).

A selective number of V. parahaemolyticus strains were tested for their ability to beta-haemolysate (Kanagawa-test). Molecular analyses of species-specific or virulence-related genes on NS and BS isolates are currently conducted by the Alfred-Wegener-Institute for Polar and Marine Research (see also VibrioNet).
2.2 Faecal indicator analyses

*E. coli* and intestinal enterococci were quantified according to ISO 9308-3 (ANONYMOUS 1998) and ISO 7899-2 (ANONYMOUS 2000), respectively, as specified in the European Bathing Water Directive 2006/7/EC (ANONYMOUS 2006). For sediment samples, bacteria were extracted from the sediments as described above prior to faecal indicator analyses.

2.3 Contextual parameters

Measured contextual parameters included water temperature, conductivity, salinity, sediment grain size, sediment TOC content (both NS and BS), phytopigments, oxygen concentration, nutrients as well as zoo- and phytoplankton community analyses (BS only). In addition, wind speed, wind direction and air temperature data derived from the National Meteorological Service.

3 Preliminary results

**North Sea**

In NS samples, *V. alginolyticus* was the most frequently occurring *Vibrio* species at all sampling sites (94 % of sediment samples, 79 % of water samples positively tested), followed by *V. parahaemolyticus* (67 % of sediment samples, 44 % of water samples). Both organisms were detected more often in sediments than in the water and were significantly correlated to water temperature (Spearman correlation *V. a.*: 0,52, *V. p.*: 0,44; p<0,001). This relationship was stronger in water than in sediments (Spearman correlation *V. a.*: 0,48, *V. p.*: 0,4; p<0,001) and our results suggest a protective effect by the sediments at cold temperatures. Approximately 6 % of *V. parahaemolyticus* strains were Kanagawa-positive.

*V. vulnificus* was detected in ~5 % of all water and sediment samples. Positive proofs concentrated mainly on the estuarine sites with salinities <25 ‰, with the exception of one positive proof on the island of Borkum at a salinity of ~30 ‰ (only sediment) (Figure 2).

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**Figure 2:** Location of the 10 sampling sites at the German North Sea coast. Red dots mark sites with positive *V. vulnificus* proofs. Blue dots mark sites where *V. vulnificus* was not detected.
*V. vulnificus* was positively related to water temperature, particularly in water samples (Spearman correlation water: 0.44; sediment: 0.29; p<0.001). *V. vulnificus* could not be detected until water temperatures reached 20 °C. After reaching this threshold, detectable concentrations could be found for longer time periods even at water temperatures as low as 14 °C (Figure 3). *V. cholerae* was of minor importance in NS waters. Only 2 % of water samples and 4 % of sediment samples were positively tested without any apparent dependency on temperature or salinity.

![Figure 3: a) Impact of water temperature and salinity on the occurrence of *V. vulnificus* in NS sediment samples. Circle sizes define concentration classes (CFU/100 g sediment). Positive proofs were reduced mainly to salinities <25 ‰ and water temperatures between 14 - 24 °C (red circle), with the exception of one positive proof on the island of Borkum (>30 ‰). B) Seasonal distribution of the number of positive *V. vulnificus* proofs in NS water and sediment samples. Blue line: mean water temperature of all sampling sites.](image)

Generally, *Vibrio* concentrations were elevated by about one order of magnitude in the sediments compared to water samples. Regarding the less important impact of water temperature in sediments compared to water, this suggests a possible role of sediments as a reservoir for potentially pathogenic *Vibrio* spp. in NS waters.

**Baltic Sea**

Compared to the NS study area, *V. alginolyticus* and *V. parahaemolyticus* played only a minor role in BS waters. *V. parahaemolyticus* was only detected at three out of twelve sampling sites (2 % of water samples, 7 % of sediment samples positively tested), *V. alginolyticus* was detected only at eight sampling sites (12 % of water samples, 17 % of sediment samples positively tested). Positive proofs mainly concentrated on October 2010 and August 2011.

*V. vulnificus* and *V. cholerae* (non-O1, non-O139) were a much more important part of the *Vibrio* community in BS waters (21 % and 31 % positive, respectively) and sediments were twice as often positively tested as waters (48 % and 57 % positive, respectively). Also, bacterial concentrations were generally one to two orders of magnitude higher in sediments than in water samples. While *V. vulnificus* occurred more frequently in the Greifswalder Bodden, *V. cholerae* was more often detected in samples of the Darss-Zingster Bodden (Figure 3).
Summer samples were much more often positive for *V. vulnificus* than spring or autumn samples, however regarding trends in NS waters it was quite surprising to find *V. vulnificus* in spring at temperatures <9 °C at all, particularly because the winter 2010/2011 had been very harsh and long. Unfortunately, we were not able to test *Vibrio* occurrence in winter, because strong ice formation both in 2011/2012 and particularly 2010/2011 hindered us from undertaking the planned cruises. Samples from April 2012 are under examination.

At this stage, we do not like to draw any conclusions concerning *Vibrio* ecology in the BS, however the environmental factors governing the occurrence of *Vibrio* in the BS will be further evaluated against the background of the available contextual data.

**Summary**

Pathogenic *Vibrio* spp. can be frequently found in German coastal waters, however the *Vibrio* communities in the German NS and BS differ quite considerably. While *V. alginolyticus* and *V. parahaemolyticus* are common members of the bacterial community in NS waters, *V. vulnificus* and *V. cholerae* are much more important in the BS. *V. vulnificus*, the most frequent causative agent of wound infections in Germany in the past, could be detected both in the NS and the BS and showed a strong relationship to water temperature. In the NS, proofs concentrated on estuarine sites. In the BS salinities are generally brackish and *V. vulnificus* could be detected over the entire study area. The link between climate change-related changes in temperature and salinity and *Vibrio* community structure needs to be further investigated.
Literature


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Warming seas and *Vibrio* disease

Craig Baker-Austin, Joaquin A. Trinanes, Nick Taylor, Rachel Hartnell, Anja Siitonen and Jaime Martinez-Urtaza

1 Introduction

Warming of coastal and marine waters may significantly extend the seasonal period and geographical range of vibrios potentially contributing to greater clinical risk (Martínez-Urtaza et al. 2010). Unfortunately, in many areas, detailed analyses regarding the epidemiology of non-cholera *Vibrio* infections are hampered by the lack of dedicated, centralised and joined-up surveillance systems (Baker-Austin et al. 2010). Areas that are now considered ‘non-endemic’ for vibrios may very soon undergo environmental transitions that can allow these pathogenic bacteria to flourish. In this regard, recent outbreaks in temperate and cold-water environments, such as Alaska (CDC 2008), Chile (Gonzalez-Escalona et al. 2005) and Northern Spain (Baker-Austin et al. 2010) underline this concern. Several large *Vibrio* outbreaks have been reported in Europe in the last 2 decades. Strikingly, these outbreaks have coincided with rapid sea surface warming across much of Europe. The focus of this presentation is to highlight the impact of recent warming trends on *Vibrio* disease outbreaks using different case studies (e. g. Galicia, Spain, and the Baltic/North Sea region), utilising a combination of available epidemiological data alongside long-term sea surface temperature (SST) and remote sensing datasets.

An analysis of surface seawater temperatures using recent satellite datasets (1982 - 2009) demonstrate rapid warming of the marine environment throughout Western Europe (Figure 1). The fastest rates of warming have occurred during summer months, with warming focussed on northern latitude areas (data not shown). These results are consistent with previous work (Belkin 2009, Lima & Wethey 2012) that indicate globally many pronounced recent summer anomalous warming patterns have been focussed in Europe. Although there are significant regional differences in both the temporal and spatial extent of surface warming, these rates of warming are, to our knowledge, amongst the most rapid identified in any global marine or coastal system. During this period of rapid warming, a large number of *Vibrio* cases have been reported, in and around Northern Europe, including outbreaks in France (Quilici & Robert-Pilloy 2011), Spain (Baker-Austin et al. 2010), and the Baltic and North Sea area (Andersson & Ekdaahl 2006, Frank et al. 2006, Lukinmaa et al. 2006).
Compiling these reports together, we identified almost 600 *Vibrio* domestically-acquired infections across Europe in the last 2 decades (including over 20 fatalities). Strikingly, most cases corresponded both temporally and spatially with extremely anomalous ‘heatwave’ periods, e. g. the years (e. g. 1994, 2003, 2006). Data derived from several studies, including those focussed on Western and Northern Europe, have identified the utility of remote sensing in delineating potential risks from *Vibrio* pathogens during such ‘at risk’ periods, which will be discussed in terms of the presented case studies from Spain and Northern Europe. However, other fundamental areas need to be addressed as part of a greater clinical understanding of these infections. Vibrios are not notifiable disease agents in Europe (with the exception of toxigenic *V. cholerae* infections), hence the data regarding the clinical impact of these diseases is often fragmentary, at best. Several issues limit this epidemiological data analysis in Europe, ranging from under-reporting, to deficiencies in laboratory practise and dissemination of epidemiological data (Figure 2). As a final discussion, we suggest and present some means of improving the reporting capacity for vibrios in Europe, based on the development of a centralised and joined-up reporting system. We propose to integrate researchers in these fields by developing an internet-based analytical platform that combines datasets and resources relevant to the entire *Vibrio* community. This centralized forum will facilitate knowledge exchange, and will provide open access to environmental and epidemiological datasets including subtyping data for risk prediction purposes. This website should include both relevant epidemiological data regarding shellfish and wound-associated cases reported worldwide, with integration of relevant datasets from CDC and ECDC, among others. Information relevant to interested researchers, such as notable outbreaks, could then be disseminated quickly via weekly bulletins.
The enigma of underreporting. The vast majority of Vibrio cases in Europe are not identified to the international Vibrio community, often because of factors ranging from self-limiting infections through to dissemination of case data in national and international epidemiological networks. Thus cases that are reported or visible internationally often represent the ‘tip of the iceberg’.

2 Summary

The emergence of non-cholera Vibrio diseases, particularly in geographical regions with a lack of long-term epidemiological datasets provides startling practical challenges to the Vibrio research community. Vibriosis is a neglected disease in Europe, in part because there is a perception that these diseases are so rare as to represent a low clinical burden in this region. However, rapid warming of coastal regions, such as that seen in and around Europe is changing the dynamics of these infectious diseases. Key areas of future work include a greater ability to track and predict these infections using remote sensing approaches, coupled to improvements in the epidemiology of Vibrio disease.

Literature


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Biological corridors as routes for the transoceanic spreading of *Vibrio* diseases

Jaime Martinez-Urtaza, Ronnie G. Gavilan and Joaquin Triñanes

1 Introduction

*Vibrio parahaemolyticus* is a halophilic marine bacterium and a natural inhabitant of estuarine areas worldwide. Environmental populations with virulence traits represent a marginal fraction of the populations naturally occurring in marine ecosystems. Under some specific environmental conditions, these rare pathogenic bacteria can cause infections in humans through the consumption of raw or undercooked marine products. The ecological determinants and environmental drivers favoring the occurrence of pathogenic groups and the emergence of infections remain yet undetermined, limiting the advances in understanding the epidemic dynamics and routes of transmission of these environmental human pathogens.

*V. parahaemolyticus* disease has traditionally been characterized by the emergence of locally occurring seasonal infections in the warmer months of the year. In 1996, an atypical increment in *V. parahaemolyticus* infections was detected in India and linked to strains belonging to a clonal group of the serotype O3:K6 (OKUDA et al. 1997; CHOWDHURY et al. 2000). Infections caused by the O3:K6 clone spread throughout the majority of Southeast Asian countries within a single year (OKUDA et al. 1997), arriving to Peru in June of 1997 (MARTINEZ-URTAZA et al. 2008) and subsequently in Chile by the end of the same year (GONZALEZ-ESCALONA et al. 2005). From this point, the pandemic clone began a constant global dissemination (NAIR et al. 2007). The routes of dissemination of the pandemic clone have been controversial from their origin. One of the most recurrent explanations has been based on the discharges of ballast waters from ships travelling from areas of *V. parahaemolyticus* endemicity. Ballast water discharges have been recognized as one of the major vehicles for the worldwide dissemination of marine species and have been identified as a reliable mechanism for the propagation of pathogenic vibrios (DEPAOLA et al. 1992).

However, bacterial dispersal through ballast water fails to provide a consistent and comprehensive explanation for the emergence of some epidemic episodes of *V. parahaemolyticus*, especially where infections have emerged in remote areas of the world without any potential source of pathogenic *Vibrio* and in those cases were cases suddenly appear simultaneously over hundreds of kilometers of coastline. In recent years, increasing evidence has related major epidemic outbreaks of *V. parahaemolyticus* to the incursion of oceanic waters of subtropical origin in coastal areas (BAKER-AUSTIN et al. 2010; MARTINEZ-URTAZA et al. 2010). The emergence and spread of infections in Peru, Spain, Chile and Alaska occurred concurrently with the arrival and movement of warm oceanic waters along the coast.
2 Results

The South Equatorial Current flows westward connecting areas of South America and South East of Asia. On the other hand, the manifestation of El Niño episode in this region implies the inversion of the dominant westward oceanic currents in the Equatorial Pacific Ocean, displacing warm and less saline waters from the Western Pacific to the coasts of South America. We have investigated over the last years the existence of possible interconnections and population admixture between *V. parahaemolyticus* populations from both sides of the Pacific Ocean and evaluated the potential role of the major marine currents in the long-distant transport of pathogenic *Vibrio* and in the spread of diseases.

The origins and routes of dissemination of the pandemic *V. parahaemolyticus* in its arrival to South America in 1997 remained unknown until recently. Population genetics analysis using VNTR of pandemic strains from Asia, Peru and Chile identified the existence of two groups within this clone. The first subgroup included strains with genotypes almost identical to Asian strains identified over the arrival of this clone to the Pacific coasts of South America in phase with the 1997 El Niño episode. The second subgroup was comprised by specimens exclusive to South America with few or no genotypic similarities with Asian strains, and included a group of Peruvian strains along with all the Chilean strains isolated in Puerto Montt (Chile) in 2004. The analysis of the surface seawater temperature data obtained by satellite in the days leading to the emergence of infections in Chile in 2004 showed the poleward progression of warm water from the South of Peru toward Chile that reached Puerto Montt in phase with the rise of infections.

MLST analysis of clinical strains isolated from infections in Peru over the last 15 years was performed to prospect additional genetic associations between Asian and South American populations. These analyses revealed additional evidences of genetic associations between Asian and Peruvian populations. Strains belonged to the O3:KUT serotype isolated in Peru over the course of an outbreak of illness in 2009 were identified as a novel clone in the country. Comparative MLST analysis of Peruvian strains and the sequences stored in the MLST public database showed that the sequences of the Peruvian strains matched with strains recently isolated in China. Additional analyses of strains of the serotype O4:K8 isolated over the last 15 years showed conserved sequences in the 7 housekeeping genes of all the strains until 1996. A novel region was detected inserted in the recA gene of strains belonging to this serotype after 1996. This distinctive and particular genetic variation in the sequence of the recA gene was also observed in strains isolated in Asia before 1995.

These results provide novel evidences of the existence of indistinguishable subpopulations of *V. parahaemolyticus* in both sides of the Pacific Ocean. These genetically related strains have been detected in distant regions interconnected by the marine currents. According to these evidences, ocean currents and the movement of waters may be playing the role of natural conduits for the long-distance dispersion of *Vibrio* specimens, with the corresponding impact on local demography and on the epidemiology of *Vibrio* diseases.
Literature


CHOWDHURY, N. R., S. CHAKRABORTY, B. EAMPOKALAP et al. (2000): Clonal dissemination of *Vibrio parahaemolyticus* displaying similar DNA fingerprint but belonging to two different serovars (o3:k6 and o4:k68) in Thailand and India. *Epidemiology and Infection*, 125, 17-25.


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Ecology and virulence of *Vibrio* spp.: some changes under the climate change influence?

Aurélie Touron-Bodilis

1 Introduction

Many diseases and environmental risks expected to increase in range and severity with projected climate changes are cited in international, European and French reports: emerging or re-emerging of infectious diseases, increased frequency and intensity of extreme events, deep changes of the environment. Phenomena influenced *likely* to *very likely* by the climate change have been identified through the work of the IPCC (Intergovernmental Panel on Climate Change), indicating a worsening of future effects of infectious and diarrheal diseases and the migration of disease vectors (GIEC et al. 2008, GIEC et al. 2007). No clear trend emerges in the number of tropical cyclones, but the effect of climate change on their intensity, the rise in average global temperature and the disruption of thermal cycling and water is projected. According to world regions, the anticipated risks linked to waterborne pathogens may be associated with: increased coastal and river flooding (Asia, Australia, New Zealand, Europe, Small Island); regional changes in extreme precipitations (increase in tropics and high latitudes, decrease in the subtropics and inferior mid-latitudes); increase in river flow and runoff (high latitudes, humid tropics, United States), which can lead to deterioration of the microbiological quality of freshwater and coastal waters; or their reduction (dry regions at mid-latitudes and in the tropics); increased cycles of flood/drought (Asia) to increase in the intensity and frequency of storms (Australia, New Zealand) and increased water temperatures. Variations in global mean temperature simulated by different climate models, indicate a *likely* increase of 0.2 °C every 10 years by 2030, and an increase from 1.8 to 4.0 °C (best estimates in the SRES scenarios) for 2090 - 2099 (GIEC et al. 2008, GIEC et al. 2007). Many factors may thus directly or indirectly modify the spatiotemporal distribution and diversity of *Vibrio* strains in the environment. Changing rainfall and runoff, causing the sediment transport, changes in salinity, turbidity and nutrient availability have been identified in estuaries and coastal deltas, making these regions particularly vulnerable (GIEC et al. 2008, HUNTER 2003). Increases in sea-surface temperature and in nutriment fluxes as a result of ENSO events, have been shown to predate increases in cholera incidence in both Asia and South America (HUNTER 2003, McMICHAEL et al. 2006, HARVELL et al. 1999).

From an international perspective, including the joint Monitoring Program of WHO and UNICEF, climate experts have identified the need to acquire more data of water quality monitoring and water consumption, but also to incorporate a monthly time step to the assessments of the impact on water “stress” (currently assessed on an annual basis), making better account of regional seasonal events (including water-borne diseases). At the end of the
work recently published by the IPCC, climate change impacts on water quality in developing countries as in developed countries and on aquatic ecosystems, are poorly understood (complexity of the association between diseases and climatic factors, lack of quality health data) and still require a significant research effort (GIEC et al. 2008).

In France, several institutional and departmental documents relating to the National Plan for Adaptation to Climate Change (PNACC) reflect or evoke the issue of "Vibrio". The risk of quality deterioration of seafood associated with the increased presence of Vibrio spp., with the consequence of having to improve compliance with cold chain of these sectors has been identified (MEEDDAT 2008), and the likely increase of the risk by extending the environmental reservoirs due to global warming of estuarine waters (PASCAL 2010). A ministerial working group (contributors IRD/DGS/AFFSET) also recommended a long-term surveillance of phytoplankton blooms and their geographical distribution, building on existing networks (REPHY, French Institute for Research and exploitation of the Sea), to anticipate the health risk associated with pathogens (including vibrios), and transmitted through the consumption of shellfish and/or fish (MEEDDAT 2010). However, the main conclusions of the national health authorities have shown that the expected impact of climate change does not justify the development of new monitoring devices in France, but rather the strengthening of existing systems and interdisciplinarity in risk management more comprehensive and inclusive. In particular, it would be necessary to have a better consistency of environmental (environmental contamination) and health monitoring systems (e.g. characterization of exhibitions and exhibitors behavioral change), but also to strengthen the knowledge of the influence of bioclimatic variables on infectious agents (virulence) (MEEDDAT 2010).

2 Climate change and potential effects on the virulence of vibrios

The health risk associated with pathogenic vibrios in marine and estuarine waters and potentially increased by climate change can be considered at two levels of knowledge. The first level is the well demonstrated today, the relationship between the increased incidence of infections in the population (due to extreme events or seasonality of the species), and increasing concentrations of cultivable Vibrio spp. in waters. These increases followed directly or indirectly to a temperature rise, or even a spatial redistribution of pathogenic strains (floods, hurricanes), and a change in behavior but also expology exhibitors (degraded access to water, water activities, seafood consumption) (QUILICI & ROBERT-PILLOT 2011). Many studies have been published for the Vibrio cholerae O1 and O139 and non-cholera species (QUILICI & ROBERT-PILLOT 2011, PARDO SEDAS 2007, GENESTE et al. 2000, QUILICI et al. 2003, ALAM et al. 2006, CHOWDHURY et al. 1992, COLLIN & REHNSTAM-HOLM 2011, COLWELL & HUQ 1994, EILER et al. 2006, HERVIO-HEATH et al. 2002, HUQ et al. 2005, LIPP et al. 2002, LOUIS et al. 2003, VALDESPINO & GARCIA-GARCIA 2011, VEZULLI et al. 2009, VEZULLI et al. 2010b, ZO et al. 2008, PASCUAL et al. 2002, MARTINEZ-URTAZA et al. 2010). A significant increase in non-cholera vibrios was also observed in Northwest Europa in recent years, and a 44-years environmental survey in southern North Sea has shown a significant relationship between the relative abundance of Vibrionaceae and of V. cholerae in the planctonic fraction and an increase of annual mean SST of 0.6 to 0.8 °C per decade and above 18 °C (VEZULLI et al. 2012).

The second level concerns the direct and indirect effects of climate change on the ecology and especially the virulence of vibrios in the environment. Within the climate range, many other social, economic, behavioural and environmental factors also affect disease occurrence.
To make a quantitative attribution of change in incidence to any single factor is therefore difficult. However, lack of knowledge of these effects has been raised by health organizations, including other pathogens. On *Vibrio* spp., many studies contribute to understanding the link between the maintenance and expression of virulence factors and environmental parameters. Some examples are given there, according to virulence mechanisms considered.

### 2.1 Potential effects on adhesion mechanisms and multiplication

It has been demonstrated that an increase in temperature might enhance *Vibrio* spp. capability to attach to and multiply on plankton, and then their capacities to colonize environmental and hosts surfaces (Stauder et al. 2010, Huq et al. 1984). Thus, the virulent potential of genes coding a mucinase in *V. cholerae* and involved in the degradation of substances similar to mucins in plants and algae has been evoked during intestinal colonization (Lipp et al. 2002). Moreover, similarly to its role during intestinal colonization, TCP may mediate bacterial interactions during biofilms differentiation on chitinaceous surfaces. Concerning animal pathogens, temperature also modulate the adhesion of *V. shiloi* on corals surface (Rosenberg & Barash 2006). The demonstration of this type of ecological and functional links is of primary importance to support the models of selection of virulence factors during colonization on environmental surfaces by *Vibrio* spp. (copepods, algae ... etc) and their potential role during human infections.

### 2.2 Potential effects on the regulation of virulence gene expression

Hayes et al. (2001) proposed a functional link between marine outbreaks and climate variability based on the Fe availability in the ocean. The relationship between Fe availability, host immunity and pathogen virulence is well-established comprising *Vibrio* spp. (Lipp et al. 2002) and since atmospheric dust should increase with the climate change, these inputs could represent the major pathway for delivering new micronutrients (Fe, Mg, Mn, Zn, Cu, Mo, Si, Co ...) to surface layers in the ocean. These large-scale increases could then alter nutrient limitation and then lead opportunistic and potential pathogens to arise and then maintain and express virulence factors as exotoxines, specific outer-membrane receptors and siderophores. Moderate levels of introduced iron also increase the expression of CT (Lipp et al. 2002). Climate influences on interactions between *Vibrio* strains population and various fauna and flora was also suggested as phenomena that could modulate virulence genes expression and/or transfer. Then, for example, possible *V. parahaemolyticus* adaptation to mammalian gastrointestinal conditions in sea otters could lead to select more virulent strains for humans (Martinez-Urtaza et al. 2010). In laboratory experiments designed to reproduce a coral disease, it has been demonstrated that virulence (tissue necrosis) of *V. harveyi, V. coralliilyticus* and *V. crassostrea* strains and maybe host susceptibility both increased significantly with tested temperatures (from 18 °C to 25 °C) (Vezulli et al. 2010a, Bally & Garrabou 2007). Moreover, expression of virulence genes in *V. coralliilyticus* has been recently demonstrated to be dependent and stimulated by increased temperature (Kimes et al. 2012). The coral pathogen *V. shiloi* has also shown a temperature-regulated production of several virulence factors, comprising hemolysis, motility, cytotoxicity and defense mechanisms against oxygen radicals (Rosenberg & Barash 2006, Murali et al. 2010). The role of salinity has also been demonstrated on the expression of CT, being optimal between 2 and 2.5 psu (Lipp et al. 2002). Moreover, the ecological role of CT has been hypothesized being an osmoregulator of the epithelial cells of the copepod, and playing the same detrimental role in human epithelial
cells leading to diarrhea (COLWELL 1996). Regulation of transcriptional activation of virulence genes can be complex and influenced by environmental factors at different levels as shown for *V. cholerae* (ToxR/S, TcpP/H and ToxT system) (SCHUHMACHER & KLOSE 1999, SKORUPSKI & TAYLOR 1197, PROUTY & KLOSE 2006), leading to the need of understanding all those regulation mechanisms before using them in infectious predictive models.

2.3 Potential effects on virulence genes transfers

The role of horizontal gene transfers and recombination events between strains (toxigenic or not) or with phages has been demonstrated in the emergence of toxigenic strains of *V. cholerae* (e. g. CTXΦ, O139 clone) and *V. mimicus* (e. g. CTXΦ). Moreover, the distinct emergence of 3 clones of *V. cholerae* O1 (classical, El Tor and US Gulf coast isolates), also supports the hypothesis of important recombination in the environment. Environmental conditions such as sunlight exposure, pH and temperature have been shown to influence the CTXΦ induction (LIPP et al. 2002). Seroconversion of non-O1 to O1 and vice-versa has also been demonstrated in laboratory experiments under variation of environmental factors such as: salinity (occurred around of ~10 psu), temperature (near 35 °C) (LIPP et al. 2002) and the presence of chitin, as an inducer of competence for natural transformation (BLOKESCH & SCHOOLNIK 2007). Moreover, conjugation assays on *V. parahaemolyticus* environmental strains lacking TDH and TRH, have demonstrated the ability of environmental strains to easily regulate new acquired virulence genes in response to temperature (MAHONEY et al. 2010). As the combination of genetic and ecological factors can lead to the emergence of new pathogenic bacteria in environmental reservoirs, it is of importance to consider the potential increase of those links under climate change.

3 Microbial and man-made worlds: complex systems to model

Understanding the co-evolution of pathogens and their hosts also means taking into account the evolving and adaptative capacities of each. Given the fact that generation time of bacteria such as *Vibrio* spp. are orders of magnitude shorter than those of their human or animal hosts, one can expect that this aspect will have to be taken into account. KOELLE et al. (2005) have proposed a model in which they have integrated an adaptative dynamic to address the evolution of the pathogen’s sensitivity to seasonal drivers (from a seasonal “specialist” to a seasonal “generalist” pathogen phenotype). Then, their approach helps to determine the sensitivity of seasonal pathogen transmission rates (e. g. *V. cholerae*) to environmental variability (KOELLE et al. 2005). Furthermore, the actual predictions of waterborne diseases can only be made for those organisms for which some data and knowledge are available. The number and diversity of other potential pathogens present in the environment that could increase and emerge under right conditions resulting in emerging infectious diseases, and the variability of host shifts is actually unknown. In a review, LEBARBENCHON et al. (2008) explained that infection networks may show ‘small world’ connections, as modern social networks, meaning that human activities had reduced the long-distance connections between geographical areas and individuals, leading to isolation of e. g. the same pathogen genotype from two distant regions of the world. Some other works showed that infectious diseases spread more easily in a small-world network than in “regular lattices”, having strong consequences for pathogens dispersal, interactions and consequently on the evolution of their virulence and resistance.
These ideas can also be applied to the many different species or ecotypes present in the genus *Vibrio*. One other example, at the microbial scale, is the quite complex temperature-dependent antagonistic interactions between marine bacteria strains and *V. cholerae* (LONG et al. 2005). It is then very important to acquire more ecological data by sampling and analysis strategies leading to the best overview of those bacteria and their natural genetic and functional diversity. Above these ecological considerations, and in the perspective of developing models and tools for public health policies, it is evident that a big challenge for researchers will be to take into account the joint evolution of man-made world and “pathogens-world”. Moreover, this should be in correspondence with the need identified by climate experts to collect health and environmental data at scales of time and space to develop appropriate public health policies (MEEDDAT 2010).

**Literature**


MEEDDAT (2010)


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Dynamics of *Vibrio* spp. in relation to phytoplankton community composition and environmental conditions

Ann-Sofi Rehnstam-Holm and Anna Godhe

**Introduction**

*Vibrio* spp. are common marine bacteria which have a broad metabolic range and can produce a variety of enzymes that enable them to use different available nutrient resources (THOMPSON & POLZ 2006). Elevated abundances of vibrios are frequently linked to temperatures above 17 °C, low salinity and eutrophication. Vibrios has also repeatedly been shown to colonize live and decaying zooplankton (HUQ et al. 1983), diatoms (ASPLUND et al. 2011, REHNSTAM-HOLM et al. 2010), corals (CERVINO et al. 2004), shellfish and fish (DEPAOLA et al. 2003, SARKAR et al. 1985), and to form biofilms (YILDIZ & VISICK, 2008).

*Vibrio* species with the capacity to infect humans, like *V. cholerae* and *V. parahaemolyticus*, are well adapted and can respond fast to elevated nutrient levels and water temperatures and have a strong chemotaxis towards ecologically relevant compounds. However, studies are largely lacking that focus on more complex environmental factors that can regulate *Vibrio* species abundances in tropical and temperate areas. Further, the characteristic virulence determinants of *Vibrio* spp., isolated from wound infections in humans, have not yet been identified.

We here briefly report some studies regarding phytoplankton-bacterial interactions, in the environment as well as in experimental systems with less complexity. We have detected and used non-O1/O139 *V. cholera* and *V. parahaemolyticus* as model organisms.

**Measuring principals**

Three diatom species (*Skeletonema tropicum*, *Ditylium brightwelli*, *Thalassiosira pseudonana*) and one dinoflagellate (*Prorocentrum micans*), and a non-pathogenic environmental *Vibrio parahaemolyticus* isolate were used in microcosm experiments. We conducted two different experiments; in experiment one we investigated the effect of temperature (15 °C and 21 °C), algal phyla (represented by two different phytoplankton species), and algal densities on the growth of *V. parahaemolyticus* (Figure 1), and in experiment two we studied the effect of diatom species richness on the abundance of *V. parahaemolyticus* estimated by a real-time PCR based 16SrDNA detection assay (Figure 2., ASPLUND et al. 2011). The second experimental setup consisted of single algal species, or combinations of two and three species in
triplicates. The total algal biovolume per mL was equivalent in all treatments. When the algal culture had reached early exponential phase, the experiment started by adding *V. parahaemolyticus*. *Vibrio* abundance was followed as CFU on TCBS agar plates.

In an environmental study we estimated the abundances of *V. parahaemolyticus* by real-time PCR in relation to total *Vibrio* spp., plankton community composition, and a range of environmental variables in the coastal water of SW India (Figure 3). Our survey was conducted during two distinct periods when the water column was stable in terms of temperature and salinity.

![Figure 1](image1.png)  
**Figure 1:**  
Maximum *V. parahaemolyticus* growth rate in different algal cultures with high and low.

![Figure 2](image2.png)  
**Figure 2:**  
*V. parahaemolyticus* abundance in single algal cultures, two combined algal cultures or all three algae in the cultures.

![Figure 3](image3.png)  
**Figure 3:**  
Coefficients plot of PLS regression models. A. *Vibrio parahaemolyticus* abundances as response variable B. *Vibrio* spp. abundances as response variable over two sampling periods (December 2007 and February-March 2008). Physical, chemical, and biological predictor variables in the model are ranked from left (most important) to the right (least important).
Summary

Algal species and biomass concentration significantly affected the maximum growth rate of *V. parahaemolyticus*, while temperature did not. Bacterial maximum growth rate was significantly higher when incubated with a diatom compared to a dinoflagellate.

Significant differences in the abundance of *V. parahaemolyticus* could only be found between the three levels of species richness.

The environmental study demonstrates temporal variation in the abundances of pelagic *V. parahaemolyticus* in an oligotrophic tropical coastal marine area (Mangalore, India, Arabian Sea), despite stable water temperatures and salinities. The number of *V. parahaemolyticus* was higher during the first sampling period in December compared to February. The most important environmental parameter coinciding with high *V. parahaemolyticus* abundances was phosphate and copepods.

Literature


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Finding the needle in the hay – targeted molecular methods for ecological studies of vibrios

Alexander Eiler and Stefan Bertilsson

Introduction

Vibrios, such as *V. cholerae*, *V. parahaemolyticus* and *Vibrio vulnificus*, are commonly found in different aquatic environments, and can cause infections in humans. The biogeographic patterns of different vibrios along natural environmental gradients, such as temperature, salinity and concentrations of organic and inorganic nutrients are not well understood. Notably, most existing studies in this field have relied on culture dependent methods for quantification and detection which only target a small (culturable) subset of the total *Vibrio* community. This limitation may be particularly severe in systems characterized by low nutrient availability and low temperature.

Culture independent molecular approaches have recently revealed that many *Vibrio* spp. maintain stable populations in high latitude waters (i.e. EILER & BERTILSSON 2006), but there are so far very few attempts to reliably detect and quantify specific *Vibrio* populations in natural waters e.g. using multiplexed quantitative PCR (i.e. KONG et al. 2002), microarrays (PANICKER et al. 2004) or quantitative PCR combined with downstream genotyping (THOMPSON et al. 2004, EILER & BERTILSSON 2006). These targeted methods often only target a portion of the actual *Vibrio* community or only provide coarse phylogenetic resolution. On the other hand assays targeting entire communities providing high resolution genotyping such as metagenomics (RUSCH et al. 2007) and 16S rRNA gene amplicon sequencing, scarcely detect *Vibrio*-related sequences, even when massively parallelized next generation sequencing approaches are used (SOGIN et al. 2006, ANDERSSON et al. 2010; HERLEMANN et al. 2011).

Besides presenting results from studies using these methods, promising alternatives to sensitive and highly specific *Vibrio* quantification in complex samples will be discussed, using e.g. amplified single molecule detection. In addition, novel approaches to visualize complex population and community data will be presented and discussed.
Methods

A total of 19 stations situated along the Swedish coastline were sampled during the summers of 2003 and 2004. *Vibrio* populations were identified and quantified by using a Q-PCR-DGGE approach as outlined in EILER & BERTILSSON (2006). Two additional data mining surveys were carried out: A three dimensional map of the Baltic Sea microflora based on high-throughput next-generation sequencing of 16S rRNA gene amplicons (HERLEMANN et al. 2011) was screened for *Vibrio* population markers. Additionally, metagenome datasets deposited in CAMERA (SESHADRI et al. 2007) were queried for vibrios using 16S rRNA, rpoB and dnaE as markers. In addition, samples were obtained from lakes to identify sources of vibrios in freshwaters.

To reveal interactions between *Vibrio* populations, other bacteria and environmental driver variables in the complex coastal/brackish communities sampled, we applied a network modeling approach as described in EILER et al. (2012). This procedure was modified by using the maximal information coefficient (MIC; RESHEF et al. 2011) to determine relationships among vibrios and between vibrios, other microbial taxa and environmental conditions.

Summary

We report the results of two biogeographical surveys from the Baltic Sea where we relied on culture independent molecular methods to specifically obtain information about the distribution of *Vibrio* populations. These independent surveys revealed a wide distribution of different *Vibrio* populations in the Baltic including detection of *Vibrio cholerae* in the Bothnian Bay that feature average yearly temperatures below 5 °C. Sporadically, vibrios affiliated with putatively pathogenic groups could even be detected in filtered lake water. Still, in contrast to findings from the studied coastal biome, vibrios in Swedish freshwaters appear to prefer an associated lifestyle as they were more commonly detected in association with freshwater phytoplankton and zooplankton. If this association with eukaryotes is important for their proliferation in low saline conditions, still, needs to be experimentally verified. Combining *Vibrio* abundance data with environmental metadata in a network approach revealed that the apparent niche separation within the genus *Vibrio* along the natural salinity gradient in the Baltic Sea may also be influenced by alternate factors such as nutrient levels and phytoplankton dynamics. Co-occurrences with phytoplankton species differed among the different *Vibrio* populations suggesting population specific associations, that may include previously demonstrated effects of phytoplankton-derived organic carbon in sustaining heterotrophic vibrios. (EILER et al. 2007).

The introduction of network analysis provide a powerful tool to gain insights into the complex lifestyle of the numerous *Vibrio* populations featured in aquatic communities. Hypotheses about interactions and environmental preferences can be formulated by visualizing relationships in temporal and spatial data on *Vibrio* dynamics and ancillary metadata which can be identified using different algorithms (i.e. co-occurrence matrices, Spearman correlations, and MIC).
Literature


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Current status of *Vibrio* research in Finland

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1 Introduction

*Vibrio cholerae* can be divided into 2 major groups: cholera-causing strains of the serogroups O1 and O139 and non-cholera vibrios (NCVs) (SACK et al. 2004). NCVs can be found as normal free-living bacteria in aquatic environments both in water and in animal reservoir, and they have been associated with other than cholera, such as gastroenteritis, acute septicemia, and skin and ear infections.

The salinity of sea water in Finland is commonly less than 1 %. Thus, it is suitable for the proliferation of vibrios at favorable temperatures. This publication describes the status of *Vibrio* research in Finland both from clinical and environmental point of view.

2 Measuring principles

A modified method of food microbiology standard (ANONYMOUS 2007) has been used in the *Vibrio* research. The primary cultivation of the aquatic and clinical specimens, including also specimens from fish, has been made on thiosulfate citrate bile salts sucrose (TCBS) or/and blood agar. From bathing water samples presumptive *Vibrio* spp. have been assessed also on CHROMagar™ *Vibrio* plates (www.chromagar.com). Presumptive *Vibrio* colonies have been identified by partial sequencing of their 16S rRNA genes, and comparing the sequences to databases as described by BERG et al. (2008).

The typical colonies and the strains studied using molecular biological methods have been confirmed by API 20 E (bioMerieaux®, Marcy-l’Etoile, France). The isolated strains have also been subjected to serogroup analysis using O1 and O139 serogroup-specific antisera. PCR assays for the *ctxA* gene have been carried out for the clinical strains and selected bathing water strains as described by NANDI et al. (2000), with slight modifications. Pulsed-field gel electrophoresis (PFGE) has been performed using the protocol described by LUKEINMAA et al. (2003).
Summary

In 1989 - 2011, seven cholera cases were reported in Finland (unpublished data from the Bacteriology Unit of the National Institute of Welfare and Health). They were all caused by strains belonging to serogroup O1, biotype El-Tor. Six of the strains were of Ogawa and one of Inaba serotype. The infections were acquired in India (3 cases), Vietnam, Indonesia and Ghana (1 case from each country). In one case in 1998, the infection originated in Finland from mussels smuggled from Thailand to Finland.

Cases of NCV infections are rare in Finland. In 1989 - 2011 the annual number of infections caused by NCVs has varied between 1 (in 1990) and 16 (in 2006) in Finland. However, *V. cholerae* NCVs have been isolated from 122 patients from which about 10 % were from blood. Most of the strains have been isolated from cultures of pus, fecal and ear swabs.

In 2003 when the sea water was extraordinary warm during the summer (maximum 23.9 - 25.1 °C) three cases of leading to hospitalization of the patients were reported a few days after the temperature had reached its highest level (LUKinmaA et al. 2006).

One patient who had coronary heart symptoms and type 2 diabetes had been swimming in the sea several times before the infection. During the summer he had also eaten several times fish caught from the sea. Results of his blood cultures revealed a *V. cholerae* NCV strain.

In another case, a boy under two years who had tumbled to a campfire had been carried into the sea as first aid for the burns. His wound cultures were positive for coagulase-negative *Staphylococcus aureus* and *V. cholerae* NCV strains.

The third case in 2003 was the only fatal NCV infection in Finland thus far. The patient had alcoholic liver cirrhosis and stable angina pectoris, and he was hospitalized after abdominal pain for 12 hours. The death of the patient was attributed to clinically invasive infection caused by NCV, and the myocardial infarction as a contributory factor. The autopsy revealed inflamed intestines, and similar NCV strains were found both in multiple intestinal samples and in the patient’s urinary culture.

The dead patient had eaten during the previous week home salted whitefish caught from sea. All the 7 strains isolated from the patients and whitefish were proven to be *V. cholerae* NCV strains which gave negative agglutination reaction with O1 and O139 antisera. In the PCR assay they were also shown to lack the *ctxA* gene. On the basis of the PFGE profiles of the human and the fish isolates, the fish seemed not to be the vehicle for the infection. Thus, the source of the infection remained unclear. In the other two cases reported in the summer 2003, the contact to seawater through a skin wound seemed to be likely.

The first suspects that swimming water could be a causative agent for skin and ear infections caused by NCVs in Finland arised at the end of the 1980’s. During the summer 1990 in the official hygienic control of bathing waters in the capital Helsinki, 36 samples for *Vibrio* analyses were performed (Kalso et al. 1992). *V. cholerae* NCV was found in two samples. Not a correlation between the clinical and environment strains was possible to demonstrate at that time.

In a three-year project in 2003 - 2005, bathing waters in Finland were studied for causative agents of adverse health effects of swimmers reported after exposure to cyanobacteria through bathing water. In the warm summer 2003, nine *Vibrio* strains from four different
samples were isolated on blood agar. Molecular biological identification of the strains (sequencing of 850 bp of their 16S rRNA genes) showed that they had high sequence similarity (>99.5 - 100 %) with the type strains of V. cholerae ATCC14035T, V. ordalii ATCC33509T and V. mimicus ATCC33653T. In 2005, 130 presumptive Vibrio strains from 25 samples were isolated from CHROMagar™ plates. On this agar, V. cholerae / V. vulnificus are expected to form green blue – turquoise blue colonies, V. parahaemolyticus mauve colonies, and V. alginolyticus colourless colonies. Confirmation by sequencing (500 bp, 16S rRNA gene) of the isolated strains of different colour (49 strains from 11 samples), however, showed that only six of the 17 turquoise blue colonies had 97 - 99 % sequence similarity with V. cholerae ATCC14035T or V. mimicus ATCC33653T. The closest sequence matches of the other strains were Aeromonas spp. (40 strains) and uncultured, unidentified bacteria (3 strains). The sequence comparisons were run a few years ago and they shall be updated due to the rapid progress in Vibrio and Aeromonas taxonomy. Serogroup analysis, PCR assay for the ctxA gene and API 20 E assays of the selected isolates (n=22) showed that none of the V. cholerae strains belonged to serogroup O1 or O139.

Due to the poor selectivity of the CHROMagar™, it was not possible to determine vibrios quantitatively in the swimming waters. The highest number of turquoise blue colonies in a sample was 1000 cfu/ml. Assuming that the confirmation frequency discussed above (approximately one third of turquoise blue colonies are vibrios) is applicable in general, the number of NCVs in the Finnish bathing waters might reach approximately 300 cfu/ml. This is so far the only quantitative information about the level of vibrios in our waters.

In the near future, the environmental monitoring of Vibrio spp. in Finland seems to be limited to the control of ship ballast waters. The international requirements for the quality of the discharged ballast water will include absence of toxigenic V. cholerae (Jørgensen et al. 2010). Globally, ships carry 3 - 10 billion tons of ballast water in their tanks every year, and ballast waters have been identified as one of the greatest threats to the sea ecosystems worldwide through introduction of invasive marine species into new environments.

In summary, limited number Vibrio research has been conducted in Finland during recent years. The studies have shown that genotypically different Vibrio strains exist in the swimming waters along the Baltic Sea coast. None of the characterized environmental isolates has been proven to be cholera-causing. Instead, all cholera cases (n=7) in Finland during the past 20 years are of foreign origin. V. cholerae NCVs, however, can cause even fatal infections at least during warm summer months and especially for persons with chronic diseases. Control and management of ship ballast waters, a better preparedness to heavy rainstorms and rise of sea water temperature due to the global warming should, however, trigger more extensive Vibrio research and monitoring also in Finland.

**Literature**


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**Vibrio cholerae** in the Large Alkaline Lake Neusiedler See, Austria: From Ecology to Risk Assessment

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1 Introduction

*V. cholerae* nonO1/nonO139 were shown to be endemic to Lake Neusiedler See, a large alkaline Central European lake (KIRSCHNER et al. 2008), and have been causing ear or wound infections, including one case of fatal septicaemia (HUHULESCU et al. 2007). This patient was suffering from an underlying malignancy and received immunosuppressive treatments.

To assess the health risk emanating from *V. cholerae* strains for tourists performing recreational activities in the lake, a schematic model was developed, considering the concentration, ecological niches, diversity, and pathogenicity of the present strains, the input of “external” strains into the lake and the potential infection pathways to the patients (Figure 1). Several investigations were performed in the past years to fill the model with data, enabling a first estimation of the risk.

![Figure 1: Schematic model of information necessary to assess the health risk from *V. cholerae* in the Lake Neusiedler See. WWTP: wastewater treatment plant](image)

2 Materials and Methods

2.1 Study site

Lake Neusiedler See is a shallow, turbid, alkaline lake ($Z_{\text{max}}$: 1.8 m, pH: 8.5 - 9.1; salinity: 1 - 2 g L$^{-1}$) located in Eastern Austria. More than 50% of its 320 km$^2$ area is covered with reed, resulting in high concentrations of humic substances in the lake (REITNER et al. 1999). The region is the most important bird sanctuary in Central Europe at the intersection point of East-West and South-North routes of migratory birds.
2.2 Sampling
Samples for all different kinds of specimens (water, zooplankton, faeces) and investigations (direct detection and isolation of *V. cholerae*, lab experiments) were taken from two to six representative sites in the open water area and in the reed belt. In addition, a series of environmental variables was determined.

2.3 *V. cholerae* abundance
*V. cholerae* were quantified by cultivation based methods, followed by biochemical tests and multiplex-PCR (BARON et al. 2007). Alternatively, *V. cholerae* were enumerated via solid phase cytometry (SPC) applying a newly developed CARD-FISH protocol (SCHAUER et al. 2012).

2.4 Laboratory experiments
For testing specific hypotheses (association of *V. cholerae* with different zooplankton species; influence of humic substances from the reed belt on growth of *V. cholerae*; invasion potential of *V. cholerae* O1 and O139 strains into the lake) batch culture lab experiments were performed.

3 Results and Discussion

3.1 Seasonal development, planktonic growth and association with zooplankton
A seasonal study over a period of 4 years showed that culturable *V. cholerae* were significantly correlated with the presence of cladocerans but not with copepods. In lab cultures, a significant association of *V. cholerae* with the cladocerans could be found, while the presence of copepods had a negative influence on *V. cholerae* growth. Cladocerans significantly enhanced *V. cholerae* growth in the surrounding water and on their surfaces with concentrations up to $8 \times 10^5$ cells per individual (KIRSCHNER et al. 2011). Moreover, we could show that also in the absence of Cladocerans planktonic *V. cholerae* showed high growth rates, comparable to the natural bacterial population. Growth was significantly reduced in water from the reed belt due to inhibition by humic substances (KIRSCHNER et al. 2008).

3.2 Potential pathogenicity of isolated *V. cholerae* strains
With one single exception, all of the strains isolated between 2002 and 2011 ($n > 1300$) were identified as *V. cholerae* nonO1/nonO139. In no isolate the ctx and tcp genes were detected. However, virulence factors like hlyA, toxR, ompU and ompW were present in 88% to 100% of investigated strains.

3.3 Invasion potential of *V. cholerae* O1/O139
*V. cholerae* strains O1 biotype ElTor and classical as well as O139 smooth and rugose were tested for their growth potential in lake water microcosms in the lab. The experiments showed that with the exception of *V. cholerae* O1 ElTor, all strains were able to multiply to high concentrations in the lake water. Growth was higher in water from the reed belt than from the lake centre.
3.4 Quantitative data of *V. cholerae* concentrations

During a seasonal cycle *V. cholerae* concentrations were quantified in the lake water and on zooplankton from various sampling sites via cultivation and CARD-FISH combined with SPC. With CARD-FISH/SPC maximum concentrations in lake water were \(~5 \times 10^5\) cells L\(^{-1}\) and *V. cholerae* could be detected throughout the year. With cultivation, maximum concentrations reached \(~2 \times 10^5\) CFU L\(^{-1}\) and followed similar seasonal patterns. However, no culturable *V. cholerae* could be detected during the winter period. On zooplankton, significantly lower concentrations were detected than in lab experiments with a maximum of \(2.5 \times 10^4\) cells ind.\(^{-1}\).

3.5 Risk assessment concerning cholera

The fact that, up to now, no *V. cholerae* O1/O139 strains were isolated from the lake and that no isolate was positive for the ctx and tcp genes, indicates that the presence of *V. cholerae* O1/O139 in the lake and the acquisition of cholera is extremely unlikely. However, as our experiments showed, an invasion of *V. cholerae* O1/O139 strains into the lake cannot be excluded for the future. Promoted by climate change (the average water temperature of the lake has increased by 1.5 °C during the past 30 years) and increased mobility of travellers, the probability of invasion will increase.

3.6 Risk assessment concerning gastrointestinal infections

For this estimation it was assumed that the published infectious dose values for gastrointestinal infections caused by *V. cholerae* O1 and non-O1 strains are identical. This means that with concentrations of up to \(5 \times 10^5\) *V. cholerae* cells L\(^{-1}\) and a reported infectious dose of \(10^5 - 10^8\) CFU (HORNICK et al. 1971, SACK et al. 1998), 0.2 to 200 L of lake water have to be swallowed to cause infection. For hot-spots of *V. cholerae* on zooplankton with up to \(8 \times 10^5\) cells per individuum (cladocerans!, lab experiments!) and a concentration of 100 cladocerans L\(^{-1}\), swallowing of less volume of water may be sufficient to cause infection.

3.7 Risk assessment concerning ear, wound and blood infections

The infectious doses of *V. cholerae* for ear, wound and blood infections have not been investigated so far. The concentration to cause infections is, however, supposed to be much lower than for intestinal infections. As all patients had either an underlying immunosuppressive disease or a chronic local inflammatory disease, it can be assumed that few cells are sufficient to cause disease in health-impaired persons, while healthy persons are less likely affected.

4 Conclusions

In conclusion it can be stated that the risk of getting a severe disease caused by *V. cholerae* during recreational activities in the Lake Neusiedler See and other comparable environments, (like e. g. the Baltic Sea; ANDERSSON & EKDAHL 2006), is extremely low for healthy persons. The risk of cholera can be excluded for the moment, as *V. cholerae* strains carrying ctx or tcp have not been detected in the lake so far. Gastrointestinal infections from non-O1/ nonO139 *V. cholerae* have not been diagnosed till now and are unlikely in view of the large volume of lake water which has to be swallowed. Immunosuppressed persons at high risk
status, however, should exercise caution, especially in the case when having wounds. When infections are observed after a visit of the Neusiedler See or other comparable ecosystems, the possibility of an infection caused by *V. cholerae* should be taken into consideration. Furthermore, increased travel activities and temperature increase due to climate change may enhance the invasion of *V. cholerae* O1/O139 strains also into saline waters of the temperate climate zone increasing the risk in the future.

**Acknowledgements**

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**Literature**


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1/2005 Praxisorientierte und vielseitig nutzbare Fernerkundungseinsätze an der Elbe
2/2005 Die Bedeutung von Baggergutrichtlinien für das Sedimentmanagement in Flussgebieten und für den Meeresenschutz
3/2005 Anwendungen der weltweiten Sammlung von Abflussdaten des Global Runoff Data Centre (GRDC)
4/2005 Feststoffhaushalt und Sedimentbewirtschaftung – anthropogene Steuerung natürlicher Prozesse
5/2005 Erfahrungen zur Niedrigwasserbewirtschaftung

1/2006 Gewässerkundliche Untersuchungen für verkehrliche und wasserwirtschaftliche Planungen an Bundeswasserstraßen
2/2006 Wasserstands- und Abflussvorschläge im Elbegebiet
4/2006 Radiologische Untersuchungen an Bundeswasserstraßen als Teil der radiologischen Umweltüberwachung
5/2006 Messkonzepte und Modellierung in der Gewässermorphologie

1/2007 Höhenmessungen mit GPS – Status quo und Entwicklungstendenzen
2/2007 Röhricht an Bundeswasserstraßen (im norddeutschen Raum)

1/2008 Neue Wege der Schadstoffbekämpfung
2/2008 Ultraschall in der Hydrometrie: neue Technik – neuer Nutzen?
3/2008 Effektive und qualitatsgesicherte Abwicklung von Sediment-/Baggergutuntersuchungen in der WSV
4/2008 Saisonale Vorhersagesysteme in Meteorologie und Hydrologie
5/2008 Umweltauswirkungen des Einsatzes von industriell hergestellten Wasserbausteinen in Bundeswasserstraßen
6/2008 Wasserbewirtschaftung und Niedrigwasser

1/2009 Wasserstandsinformationsdienste der BfG für die Bundeswasserstraßen
2/2009 Sediment Contact Tests. Reference conditions, control sediments, toxicity thresholds
3/2009 Sedimentologische Prozesse – Analyse, Beschreibung, Modellierung
4/2009 Ingenieurvermessung im Bauwesen der Wasser- und Schifffahrtsverwaltung
5/2009 Verfahren der ökotoxikologischen (Risiko-) Bewertung in der Umweltsicherung
6/2009 Softwarelösungen für ein integriertes Hochwassermanagement
7/2009 Aspekte des Schadstoffmonitorings an Schwebstoffen und Sedimenten in der aquatischen Umwelt

1/2010 Flussysteme in Raum und Zeit
2/2010 Berücksichtigung verkehrs- und bautechnischer Emissionen und Immissionen in Umweltverträglichkeitsprüfungen
3/2010 Pathogene Vibrionen in der marinen Umwelt
4/2010 Riskobewertung stofflicher Belastungen
5/2010 Screeningverfahren zur Erfassung endokriner Wirkungen in der aquatischen Umwelt

1/2011 Erfassung und Bewertung des hydromorphologischen Zustands in Wasserstraßen
2/2011 Umweltauswirkungen von Wasserinjektionsbaggerungen
3/2011 Zeitgemäße Erfassung und Bereitstellung von Geobasisdaten für die WSV
4/2011 EurAqua Symposium Impact of climate change on water resources – 200 years hydrology in Europe – a European perspective in a changing world
5/2011 Schadstoffdynamik in Flussgebieten – Ursachen, Wirkungen und Konsequenzen stofflicher Veränderungen in Raum und Zeit

1/2012 Partikuläre Stoffströme in Flusseinzugsgebieten
2/2012 Überregionale Wasserbewirtschaftung – Entwicklung und Einsatz eines Informationssystems und verschiedener Modelle
3/2012 Dynamik des Sedimenthaushaltes von Wasserstraßen