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Long-term effects of ocean warming on the prokaryotic community: evidence from the vibrios

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Abstract

Long-term effects of ocean warming on prokaryotic communities are unknown due to the lack of historical data. We overcame this gap by applying a retrospective molecular analysis to the bacterial community on formalin-fixed samples from the historical Continuous Plankton Recorder archive, which is one of the longest and most geographically extensive collections of marine biological samples in the world. We showed that during the last half century, ubiquitous marine bacteria of the *Vibrio* genus, including *V. cholerae*, increased in dominance within the plankton-associated bacterial community of the North Sea where an unprecedented increase in bathing infections related to these bacteria was recently reported. Among environmental variables, increased sea surface temperature explained 45% of the variance in *Vibrio* data supporting the view that ocean warming is favouring the spread of vibrios and may be the cause of the global increasing trend in their associated diseases.

Introduction

The most immediate and direct effect of climate change at a global ocean scale will be the increment in the sea surface temperature (SST) that is estimated to increase by a few degrees during this century (Harvell *et al.*, 2002). Although much evidence has been accumulated on the long-term effects of ocean warming on eukaryotic populations (e.g. animals and plants) (Edwards and Richardson, 2004; Sala and Knowlton, 2006), no experimental information exists for the effects this may have on marine prokaryotic abundance and diversity (Sarmento *et al.*, 2010). An explanation for this gap is the lack of historical data and the belief that lower trophic levels, such as the primary producers (phytoplankton) and decomposers (heterotrophic prokaryotes), are considered less sensitive to environmental change than their consumers or predators, since sensitivity to climate is considered to increase with trophic level (Voigt *et al*., 2003; Raffaelli, 2004).

Vibrios are gram-negative, curved, rod-shaped bacteria belonging to the class *Gammaproteobacteria* and are still regarded by most marine microbiologists as the dominant culturable bacteria in the ocean (Pruzzo *et al.*, 2005). They are found on a number of biotic and abiotic substrates, notably associated with chitinous plankton that is considered to be an important reservoir of these bacteria in nature (Colwell, 1996; Turner *et al*., 2009). Undoubtedly, the most well-known member of the genus is *Vibrio cholerae*, the etiological agent of epidemic cholera (Kaper *et al.*, 1995). Other vibrios capable of causing disease in humans include *V. parahaemolyticus*, the agent of seafood-associated gastroenteritis worldwide (Levin, 2006), and *V. vulnificus,* the cause of septicaemia and serious wound infections as well as the leading cause of shellfish-associated deaths in the United States (Shapiro *et al.*, 1998). Several *Vibrio* species are also pathogenic towards marine animals, including molluscs (Paillard *et al.*, 2004), corals (Vezzulli *et al.*, 2010) and fish (Austin, 2005), with major economic and environmental impacts.

There is substantial evidence that *Vibrio* associated diseases are increasing worldwide with climate warming (Harvell *et al.*, 2002). For example, increased SST linked to El Niño events have been shown to pre-date increases in cholera incidence in both Asia and South America (Pascual *et al.*,

12000). Similarly, climate anomalies have been implicated in the expansion of the geographical and 2seasonal range of seafood-borne illnesses caused by *V. parahaemolyticus* and *V. vulnificus* 3(Martinez-Urtaza *et al.*, 2010). Evidence has also been gathered linking *Vibrio* infections to 4increasing mass mortality of marine life in the coastal marine environment (Paillard *et al.*, 2004; 5Vezzulli *et al*., 2010).

6The 2010-2011 MCCIP Annual Report Card (www.mccip.org.uk/arc) that provides an up-to-date 7assessment of how climate change is affecting UK seas considered, for the first time, the potential 8future increases in marine vibrios as an emergent issue (Marine climate change impacts Annual 9Report Card 2010-2011). In recent years, in a number of Northwest European countries, there has 10been an unprecedented increase in the number of bathing infections that have been associated with 11warm water *Vibrio* species. For example, during the hot summer of 2006 wound infections linked to 12contact with Baltic and North Sea waters were reported from Germany (*V. vulnificus*) (Frank *et al.*, 132006), southeast Sweden (*V. cholerae* non-O1/O139) (Andersson and Ekdahl, 2006), the 14Netherlands (*V. alginolyticus*) (Schets *et al.*, 2006) and Denmark (*V. alginolyticus* and *V.* 15*parahaemolyticus*) (Andersen, 2006). These occurrences have increased concern over the 16contribution that climate change may be making to the abundance of vibrios in coastal seas (Marine 17climate change impacts Annual Report Card 2010-2011). However, despite the volume of indirect 18evidence, it is not clear whether vibrios, which are known to be thermodependant, are increasing 19within the complex and ecologically regulated bacterial communities in coastal marine waters. This 20is mainly due to a lack of historical data.

21To assess a possible linkage between the occurrence of *Vibrio* and SST over a decadal scale, we 22applied molecular and pyrosequencing analysis to the microbial community on formalin-fixed 23samples from the historical archive of the Continuous Plankton Recorder (CPR) survey (**Fig. 1**). 24This survey has produced one of the longest and most widespread time series covering the 25abundance and distribution of marine organisms in the world (http://www.sahfos.ac.uk). The CPR 26was designed as a zooplankton sampler, but also samples, in a semi-quantitative way, smaller

components of the plankton, including mucilage and detrital particles trapped by the fibrils of the filtering silk and the reduced filtration mesh due to trapped zooplankton (Reid *et al.*, 2003). Several studies have been undertaken that compare plankton abundances obtained with the CPR with those obtained using standard plankton nets. Although catches by the CPR are almost always lower, seasonal cycles are replicated in each comparison, and interannual variability generally agrees between time series (Batten et al., 2003).

Plankton represents a nutrient-rich reservoir capable of enriching *Vibrio* species, which may be present, especially during the warmer months, at high densities (Turner *et al.*, 2009). For this reason, it is likely that the CPR system captures a substantial fraction of these bacteria and can provide a long-term record for *Vibrio* and other particle associated bacteria present in the water filtered during a tow.

We provide evidence that vibrios, including the species *V. cholerae*, increased in dominance within the plankton-associated bacterial community of the North Sea during the past 44 years and that this increase is correlated significantly with climate-induced sea surface warming during the same period.

Material and Methods

Continuous Plankton Recorder samples

The Continuous Plankton Recorder (CPR) is a high-speed plankton sampler designed to be towed from commercially operated ships of opportunity over long distances (Reid *et al.*, 2003) (**Fig. 1**). Sampling takes place in the surface layer (~7 metres) and plankton is collected on a band of silk (mesh-size 270 μm) that moves across the sampling aperture at a rate proportional to the speed of the towing ship (Reid *et al.*, 2003). The CPR mesh width of 270 μm retains larger zooplankton with a high efficiency but also collects small planktonic organisms such as nauplii, microzooplankton and phytoplankton (Batten *et al*., 2003). Over 500 phytoplankton and zooplankton taxa are routinely collected, identified and counted on CPR samples. Some are identified to the level of species, some

to genus, and some to a higher taxonomic group (see Vezzulli *et al*., 2003 for a list of major plankton taxa identified in CPR samples collected in the North Sea). On return to the laboratory, the silk is removed from the device and divided into individual samples that are numbered along the route. Only odd samples are analysed, according to standard procedures. Both analysed and unanalysed samples are stored in plastic boxes in buffered formalin (usually comprising 4–10% buffered formalin) in the CPR archive in Plymouth (England). CPR samples used in this study were collected in two areas located off the Rhine (51.9-52.4° N; 3.3-4.0° E) and Humber (53.5-54.0° N; 80.1-0.9° E) estuaries in the North Sea, in August (corresponding to the seasonal peak of *Vibrio* counts in seawater), from 1961 to 2005 (**Fig. 1**). The outer limit for sample collection in this study was defined as within 50 nautical miles of the North Sea coast.

Temperature and plankton data

Average SST time series for the Rhine and Humber regions, in summer (August), were calculated from the HadSST dataset (Rayner *et al.*, 2006) using the BADC data explorer (http://cdat.badc.nerc.ac.uk/cgi-bin/dxui.py). Yearly mean time series of the Phytoplankton Colour index (a visual index of chlorophyll, Reid *et al.,* 2003) and abundance of Total Copepods for the same areas, were calculated using the WinCPR database and associated software (Vezzulli *et al.*, 2007).

Nucleic acid extraction and purification from CPR samples

For each CPR sample, the filtering silk was cut into five replicate (1 cm^2) sections and each section was placed in a sterile tube. Twenty-five ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) were added and the sample was vortexed to detach plankton from the silk mesh. Samples were incubated at room temperature for 24h and vortexed for 30s. Each plankton suspension was gently centrifuged and the pellet transferred to a sterile microcentrifuge tube and DNA extraction was performed. Briefly, 50µl of lysozyme (2 mg ml-1 in 10mM Tris-HCl, pH 8.0) were added to the

1sample that was then vortexed vigorously for 1min. After addition of 180µl 10% SDS and 25µl
2proteinase K (10 mg ml^{-1}), the sample was vortexed for 30s. The sample was then incubated at 56°C
3for 1h, heated at 90°C for 1h in a dry-block heater, vortexed for 10s, and centrifuged at 12,000g for
43 min. After addition of 200 µl guanidine hydrochloride lysis solution and 200µl ethanol, the
5sample was centrifuged (12,000g for 10 sec). The supernatant was then transferred to QIAamp
6MinElute column (Qiagen, Valencia, California, USA) and processed according to the
7manufacturer's recommendation. The retained DNA was purified with QIAquick PCR purification
8columns (Qiagen, Valencia, California, USA) up to a final yield of 1-7 µg ml^{-1}. PCR inhibition tests
9were conducted on serially 1:2 diluted samples to which 10 copy/reaction of a genomic reference
10DNA was added.

11

12**Sizing and quantification of genomic DNA**

13The amount of DNA extracted from the CPR samples was determined fluorimetrically with
14PicoGreen using a NanoDrop® ND-3300 fluorometer (NanoDrop Technologies, Wilmington, DE,
15USA). Sizing of genomic DNA was conducted in an Agilent Bioanalyzer 2100 (Agilent, Palo Alto,
16CA) using the High Sensitivity DNA kit (Agilent Technologies).

17

18**Real-Time PCR**

19To calculate a *Vibrio* relative Abundance Index (VAI) (**Fig. 1**), 10ng of genomic DNA extracted
20from a 1 cm^2 section (for a total of five replicate sections for each CPR sample) was analysed by
2116S rRNA gene-targeted Real-Time PCR with SYBR-green detection using a capillary-based
22LightCycler instrument and a standard curve method for quantification. The oligonucleotide primers
23used in the PCR reaction were: Vib1 f-GGCGTAAAGCGCATGCAGGT; Vib2 r-
24GAAATTCTACCCCCCTCTACAG (Thompson *et al.*, 2004) specific for the genus *Vibrio* and
25967f-CAACGCGAAGAACCTTACC; 1046r-CGACAGCCATGCANCACCT (Sogin *et al.*, 2006)
26specific for the domain *Bacteria*, amplifying positions 567–680 and 965-1063 (V6 hyper variable

region) of the *Escherichia coli* numbering of the 16S rRNA, respectively. Each reaction mixture contained 5.0mmol of MgCl2 and 0.25µmol of each primer in a final volume of 20µl. The PCR programme was optimised as follows: initial denaturation at 95°C for 10min, subsequent 40 cycles of denaturation at 95°C for 5s, annealing at 58°C (Vibrio spp.) or 57°C (Total bacteria) for 5s and elongation at 72°C for 4s, followed by final elongation at 72°C for 10min. PCR runs were analysed directly in the LightCycler using melting analysis and the software provided with the instrument. For each single Real-Time PCR assay each DNA template was analysed in triplicate (coefficient of variation < 5%). Standards were prepared from 16S rDNA nucleic acid templates of *Vibrio cholerae* El Tor N16961 at known molar concentrations. *Vibrio* spp. and total bacterial concentrations were expressed as number of cells per square centimetre of the CPR sample (cells/cm^2) by dividing the total 16SrDNA copy number by the average 16SrDNA copy number in vibrios (n=9, Acinas *et al*., 2004) and proteobacteria (n=3.5, Kormas, 2011), respectively (Table S2).

Pyrosequencing

A PCR amplicon library was generated from genomic DNA extracted and pooled for a total of five replicate sections for each CPR sample using the broad-range bacterial primers, 967f and 1046r, amplifying the V6 hypervariable region of ribosomal RNAs (Sogin *et al.*, 2006). The PCR products were pooled after cycling and cleaned to a total yield of 300 ng using AMICON Ultra 30K membrane (Millipore, Billerica, MA 01821). Amplicon libraries were bound to beads under conditions that favour one fragment per bead and beads were emulsified in a PCR mixture in oil. After breaking the emulsion, the DNA strands were denatured, and beads carrying single stranded DNA clones were deposited into wells on a PicoTiter-Plate (454 Life Sciences) for pyrosequencing on a 454 Genome Sequencer FLX Titanium (Roche, Basel, Switzerland). Sequence Reads data is archived at NCBI SRA with the accession SRA026732.

Bioinformatics analysis

Raw data obtained by the 454 Genome Sequencer FLX were trimmed as described (see Results and Discussion). To assess taxonomic diversity, each trimmed read sequence was BLASTed against a reference database of ~40,000 unique V6 sequences extracted from the nearly 120,000 published rRNA genes for the Bacteria domain (Sogin *et al.*, 2006) using Blast (version 2.2.18). We performed MEGAN (Metagenome Analysis Software, version 3.8) analysis, using a bit-score threshold of 35 and retaining only hits whose bit scores were within 10% of the best score. All assignments hit by less than 20 reads were discarded.

Statistical analysis

The relationship between *Vibrio* abundance and the predictor variables (SST, Phytoplankton Colour index and Total Copepod abundance) was assessed using a non-parametric multiple regression analysis that was based on Euclidean distances calculated on normalized data using the routine DISTLM forward 1.3 (Anderson *et al.*, 2003). Forward-selection of individual variables is used, where amounts explained by each variable are added to the model and are conditional on variables already in the model.

Results and Discussion

Increase over 4 decades in the relative abundance of Vibrios with rising SST

We analysed a set of 55 samples collected by the CPR survey in the North Sea from off the Rhine and Humber estuaries between 1961 to 2005 (**Fig. 1, Table S1**). All samples were collected in August by a CPR machine and each represents a transect of 10 nautical miles (18.5 km) corresponding to 3m^3 of filtered seawater (Reid *et al.*, 2003). The total area sampled equals ~1400 square miles off the Rhine and ~1600 square miles off the Humber estuaries over a 44 year period (**Fig. 1**).

We assessed the presence and relative abundance of vibrios on CPR samples by applying a real-time PCR approach that used genomic DNA recovered by an improved extraction and modified purification methodology based on previous studies (Kirby and Reid, 2001; Ripley *et al.*, 2008). 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 (**Fig. 2**). Up to now, reliable molecular quantification of biological targets associated with these samples was considered impossible because of the sample age and storage in formalin, which is believed to hamper molecular analysis principally by causing DNA degradation and crosslinking between adjacent DNA molecules (Ripley *et al.*, 2008). It has also been shown that formalin may interfere with molecular techniques that rely upon enzyme activity such as PCR (De Giorgi et al., 1994). To overcome these concerns, we firstly assessed DNA fragmentation from historical CPR samples showing that DNA smear in the 200-800bp size range prevailed in genomic DNA recovered from the older samples, the earliest of which date back to August 1961 (**Fig. S1**). In order to assess the suitability of the extracted DNA for the molecular study of the bacterial community a number of PCR trials were then carried out on all samples using (according to DNA fragmentation analysis) primer pairs that amplify a small region (98bp) of the 16SrDNA of Vibrios (**Fig S1**). On the basis of these successful trials we finally developed an unbiased index of abundance for *Vibrio* quantification in CPR samples termed a '*Vibrio* relative Abundance Index' (VAI) (**Fig. 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 total bacterial cells assessed by Real-Time PCR using genus-specific and universal primers, respectively. According to the above results, ~100bp amplicons were used, allowing amplification to take place and maximising the reaction yields when the DNA was fragmented. In addition, PCR protocols based on similar size amplicons (113bp vs 98bp for *Vibrio* and total *Bacteria,* respectively) were employed 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 (**Fig. 3a,c; Fig. S2**).

Our results show a long-term increase in relative *Vibrio* abundance coupled to a positive and statistically significant correlation with SST off the Rhine Estuary but not off the Humber Estuary during the past 44 years (**Fig. 3e**). The differences between the two areas may be related to the generally higher summer SST values recorded in the Rhine compared to the Humber area. It is well known that most Vibrio species thrive in seawater, during the seasonal cycle, when temperature exceeds 16-18°C (Thompson *et al.,* 2004; Vezzulli *et al.,* 2009). In the Rhine area SST in summer generally exceeded 18°C especially during recent years. In contrast, off the Humber, SST values never exceeded 18°C during the entire time series (**Fig. 3e**). To investigate the long-term relationship between *Vibrio* abundance and SST we used data pooled from the two areas (n=55) in the North Sea and applied a non-parametric multiple regression analysis (Anderson, 2003). Since plankton and especially copepods represent important reservoirs of vibrios in the aquatic environment we included the number of Total Copepods and Phytoplankton Colour, calculated for the same areas and period (Vezzulli *et al.*, 2007), (**Fig. 3b,d**), as additional predictor variables in the model. Salinity ranging from 33-35 PSU was not included as no significant year-to-year change is reported in the analyzed period in the study area (Marine Climate Change Impacts Partnership Annual Report Card 2010).

We showed that in the North Sea, SST and the number of Total Copepods explained 50% of the variance in the *Vibrio* data ($p<0.05$) (**Table 1**). In contrast, Phytoplankton Colour was redundant in the model ($p>0.05$). SST alone explains 45% of the variance in *Vibrio* data supporting evidence from previous studies, that an increase in temperature might enhance not only *Vibrio* growth rates but also their capability to attach to and multiply on plankton (Huq *et al.*, 1984).

In the late 1980s an ecological regime shift occurred in the North Sea that was linked to a shift to a positive North Atlantic Oscillation (NAO) index and coincided with an increased incursion of warm oceanic water from the Atlantic into the northern North Sea (Reid *et al.*, 2001). This event affected

all trophic levels including phytoplankton, zooplankton, and benthos to fish (Reid *et al.*, 2001; Kirby *et al.*, 2007). Since approximately this shift (25 years), SST has risen in UK waters with the largest increases, of between 0.6˚C and 0.8˚C per decade, in the southern North Sea (Marine Climate Change Impacts Partnership Annual Report Card 2010). Here we show, for the first time, that warming of seawater temperature over a decadal scale also had a major impact on the prokaryotic community increasing the relative abundance of warm water vibrios associated with plankton in coastal waters. We emphasise however, that the ratio of vibrios associated with plankton versus free-living vibrios is still an open question. Although some authors (Turner *et al*., 2009; Stauder *et al*., 2010) have shown that a high load of vibrios is associated with plankton, the number of free-living vibrios may be even higher. The relative proportion of vibrios associated with plankton compared to free-living concentrations in sea water deserves further investigation especially in the light of climate change and rising SSTs.

In this study, a marked increase in *Vibrio* abundance occurred after the late 1980s off the Rhine estuary in the North Sea, matching the ecological regime shift and an associated stepwise increase in SST after 1987 (Kirby *et al.*, 2007) (**Fig. 3a**).

Vibrios increased in dominance within the plankton-associated bacterial community

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. The bacterial community composition of five selected CPR samples collected off the Rhine Estuary in 1961, 1972 and 1976 (before the regime shift) and in 1998 and 2004 (after the regime shift), was examined. We sequenced ~44,000 PCR amplicons spanning the V6 hypervariable region of the 16S ribosomal RNA gene from genomic DNA of the CPR samples. The number of reads per sample ranged from 6,037 to nearly 13,000 sequences. To minimize random sequencing errors, a stringent trimming procedure was followed by eliminating reads that contained one or more ambiguous bases

1(Ns), had errors in the barcode or primer sequence, were atypically short (less than 70bp), and had 2an average quality score less than 30 (Sogin *et al.*, 2006). On average this step reduced the size of 3the dataset by 23% resulting in a total of 10.066 (year 1961), 6.745 (year 1972), 6.014 (year 1976), 45.173 (year 1998) and 8.206 (year 2004) trimmed read counts available for bioinformatics analysis. 5To assess taxonomic diversity, each trimmed read sequence was BLASTed against a reference 6database of ~40,000 unique V6 sequences extracted from the nearly 120,000 published rRNA genes 7for the bacterial domain (Sogin *et al.*, 2006).

8The results of BLASTN were used to estimate the taxonomic content of the dataset, using NCBI 9taxonomy with MEGAN (Huson *et al.*, 2007). We had to consider that external contamination of 10CPR samples would be likely to occur during silk manufacture and removal and cutting from the 11sampling cassette. Risks associated with laboratory handling were minimised by using un-analysed 12samples. To further reduce bias, we restricted analysis of the 454 sequences to the *Alpha*- and 13*Gammaproteobacteria* that are known to be abundant in seawater (De Long, 1996; Venter *et al.*, 142004) and not usually associated with human or laboratory contamination. In addition, only the 15most abundant reads (e.g. those occurring at least 20 times in the trimmed dataset) were assigned to 16bacterial taxa and included in the results (**Fig. 4**). The lowest common ancestor (LCA) algorithm 17assigned 35,063 reads to taxa, whilst 1,138 remained unassigned, either because the bit-score of 18their matches or the minimum number of reads for taxon assignment fell below the threshold (see 19methods). A total of 33,786 reads were assigned to the domain *Bacteria*, of which 3,207 were 20assigned to the family *Vibrionaceae*.

21We used a multiple-comparative analysis in MEGAN to compare CPR pyrosequencing data from 22the different years with taxon data normalized over all reads, such that each data set had 100,000 23reads (Mitra *et al.*, 2009). This minimized potential bias arising from differences in absolute read 24counts, e.g. loss of sequences due to degradation which could be related to age and formalin storage 25of the samples. We showed that among microorganisms that correspond to the most abundant 26OTUs, a major shift in bacterial community composition occurred, that would be attributed to an

increase in the family *Vibrionaceae* including the human pathogen *V. cholerae* (**Fig. 4, Fig. S3**). This detailed taxonomic analysis revealed the presence of a number of bacterial taxa in assemblages associated with coastal CPR samples, dating back to August, 1961. These data 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. Our results open up a novel window for the long-term and retrospective study of microbial biodiversity and the global ecology of marine bacterial communities.

Conclusions

In conclusion, our analyses have demonstrated that a major change in the structure of the bacterial community that is associated with plankton occurred in the southern North Sea in response to increasing SST over the last 4 decades . This finding is intriguing as it provides a long-term pattern for environmental microbial communities, including abundance and diversity of bacterial species that, despite being dependant on a complex interplay between biotic and abiotic factors in both large and local-scale processes, appears to be driven largely by sea surface warming. Based on this evidence, the increasing dominance of marine *Vibrio* spp., including pathogenic bacterial species, among plankton associated bacterial communities of coastal seawater may very likely occur in other areas around the world as a response to climate change. Potential effects on human and animal health and ecosystem functioning are at present unpredictable.

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Author Contributions

Author contributions: L.V., C.P., I.B., M.G.H., P.R. and R.C. designed research; L.V., I.B., E.P. performed research; L.V., C.P., E.P., I.B., M.G.H., and R.C. analyzed data; and L.V. and C.P. wrote the paper. Correspondence and requests for methods and figures should be addressed to L.V. (luigi.vezzulli@unige.it).

Supplementary information is available at The ISME Journal's website

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Titles and legends to figures

Figure 1. Retrospective assessment of relative abundance of vibrios (VAI index) in the North Sea

Methods used to calculate relative abundance of vibrios (VAI index) from formalin-fixed plankton samples collected by the Continuous Plankton Recorder (CPR) survey off the Rhine and Humber estuaries, in August, from 1961 to 2005. **a.** CPR samples were collected in the North Sea from 1961 to 2005 by the Continuous Plankton Recorder survey. **b.** back in the laboratory the silk containing the entrapped plankton (under 4–10% buffered formalin) is cut into blocks each representing 10 nautical miles of tow (3m^3 of filtered seawater). **c.** 5 replicates 1cm^2 sections are prepared from each CPR block for microbiological molecular analyses. **d.** genomic DNA is extracted from each section and purified. **e.** the ratio of *Vibrio* spp. cells to total bacterial cells is assessed by Real-Time PCR from 10ng of genomic DNA from each section using genus-specific and universal primers respectively, producing small amplicons of similar size (113bp vs 98bp) to avoid age and formalin induced bias (see main text). The *Vibrio* relative Abundance Index (VAI) is then calculated for each sample by averaging values of this ratio for the 5 replicate measurements.

Figure 2. PCR-based amplification of bacterial DNA extracted from historical CPR samples back to August 1961

Melting curve analysis (A) and agarose gel (B) showing output of real-time PCR amplification of a 113bp DNA fragment targeting the 16SDNA gene of *Vibrio* spp. from genomic DNA extracted from archived formalin-fixed CPR samples from the North Sea off the Rhine estuary back to August 1961.

Figure 3. Relative abundance of *Vibrio* spp. and levels of environmental variables

Long term variation in the abundance of *Vibrio* (a,c) (blue triangles) (error bars indicate standard deviation, n=5), SST (a,c) (red circles), Phytoplankton Colour index (b,d) and Total Copepods (b,d) for 1961 to 2005 off the Rhine and Humber estuaries in the North Sea. Vertical red line = regime shift step change in temperature after 1987 (Kirby *et al.*, 2007). Horizontal blue lines = Average standardized VAI values for the two periods, –0.94 SD (1961 to 1976) and +0.4 SD (1989 to 2005) for the Rhine area and –0.13 SD (1965 to 1987) and -0.13 SD (1988 to 2005) for the Humber area. (e) Pearson correlation analysis between *Vibrio* abundance and SST in the North Sea (Pearson correlation on pooled data; n= 55; r=0.27*; $p < 0.05$). Z values are obtained by subtracting the population mean and dividing the difference by the standard deviation.

Figure 4. 16S rDNA pyrosequencing-based comparative analysis of the microbial community from historical CPR samples

Comparative analysis of 16S rDNA pyrosequencing data for dominant *Alpha-* and *Gammaproteobacteria* groups are shown for CPR samples collected in 1961, 1972, 1976, 1998 and 2004 off the Rhine Estuary. A heat map is shown where the numbers of normalised reads taken by each taxon in each year are represented as colours (cold-to-hot colour representing low to high number of reads). The cumulative number of normalized reads across the different years is also shown for each taxon (Mitra *et al.*, 2009). BR= before Regime shift; AR= after Regime shift. a) The tree is collapsed to the 'Family' level; b) Results for the *Vibrio* genus, VAI index is reported for comparison; c) Number of normalized read sequences showing >95% identity to *V. cholerae* (see also **Fig. S3**).

Table 1.

Long-term relationship between vibrios and environmental variables in the North Sea

Results of a multiple regression analysis of the *Vibrio* relative Abundance Index data versus SST, the Phytoplankton Colour index and Total Copepods in the North Sea. Outputs from the analyses include: (**a**) the results of the marginal tests (i.e. fitting each environmental variable individually, ignoring the others), followed by (**b**) the results of the forward selection procedure with the conditional tests (i.e. fitting each environmental variable one at a time, conditional on the variables that are already included in the model). The multiple regressions were based on Euclidean distances calculated among observations from normalised data. The forward selection of the predictor variables was done with tests by permutation. P-values were obtained using 4999 permutations of raw data for the marginal tests (tests of individual variables), while for all conditional tests the program uses 4999 permutations of residuals under the reduced model. %Var: percentage of variance in *Vibrio* data explained by that individual variable and Cum %: cumulative percentage of variance explained. *p<0.05

Set	%Var	pseudo-F	P	Cum (%)
a) Marginal tests				
***Vibrio* abundance vs.**				
SST	45.4	42.4	0.0001	
Phytoplankton Colour	33.4	25.6	0.0002	
Total Copepods	20.5	13.2	0.0004	
b) Sequential tests				
***Vibrio* abundance vs.**				
SST	45.4	42.4	**0.0001***	**45.4**
Total Copepods	4.2	4.17	**0.04***	**50.0**
Phytoplankton Colour	1.9	1.96	0.17	51.5