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Global Biogeography and Ecology of Vibrio in a Warming Planet

by

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"You steer the ship the best you know. Sometimes it's smooth. Sometimes you hit the rocks."

--C.S.

“Fortune favors the prepared mind.”

--L.P.

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Abstract

This doctoral thesis explores the broad-scale patterns, biogeography, and ecology of *Vibrio* species across the world's oceans through the molecular analysis of marine plankton samples and the use of large metagenomic data derived from global ocean sampling efforts such as the Continuous Plankton Recorder (CPR) survey and the TARA Ocean Expedition. The *Vibrio* genus, including clinically important pathogenic species such as *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. alginolyticus*, plays a significant role in aquatic ecosystems and human health. Through deep metagenomic shotgun sequencing and the global oceanic samplings of the TARA Ocean expedition, approximately 40 terabases of data from 1,500 TARA metagenomes were analyzed, employing a custom bioinformatics pipeline. The pipeline permits to download, extract, and analyze *Vibrio* reads from complex metagenomic data. This approach enabled a detailed examination of *Vibrio* diversity, biogeography and dispersal patterns via ocean currents and their ecological interactions within major biological corridors in the ocean as a planetary interconnected ecosystem (see chapter 3). Results showed that *Vibrio* represents a major player in the oceans being the 7th most abundant group in the oceans with differentiated communities living in the water fraction and attached to plankton. While free-living communities showed a well-defined biogeographical pattern, plankton-attached populations showed a strong association with small (20-180 µm) plankton, which governed its distribution and connectivity across the oceans following the major migratory routes. These findings have strong implications for the demography, population dynamics and evolution of *Vibrio* species, including those species pathogenic for humans and animals.

In addition, in this thesis, the large-scale impact of the 2016 marine heatwave (MHV) on plankton associated *Vibrio* communities was investigated along an ~800 km transect in the Great Barrier Reef (GBR, Australia) from November 2014 to August 2016 (see chapter 4). Novel methodologies for pathogen detection in CPR samples collected in the GBR by the Australian Continuous Plankton Recorder (AusCPR) were developed. 16S rRNA gene metabarcoding of archived plankton samples were applied showing a significant increase in bacteria belonging to the genus *Vibrio* during and after the 2016 heatwave. Notably, Droplet Digital PCR and targeted metagenomic analysis applied on samples collected four months after the MHW event revealed the presence of several potential pathogenic *Vibrio* species associated with diseases in aquatic animals. Overall, the 2016 MHW significantly impacted the surface picoplankton community and fostered the spread of potentially pathogenic bacteria across the GBR providing an additional threat for marine biodiversity in this area. These efforts, integrating extensive sequencing analysis with environmental data, illustrate how large and global scale concepts can help integrate *Vibrio* biological complexity in relation to climate change and oceanic conditions; as well as for assessing ecosystem changes for our planet in the Anthropocene epoch.

Chapter 1: General Introduction:

A) Milestones in *Vibrio* history: From Ancient Origins to Modern times

Abstract:

There was a time in human history when infectious diseases used to wipe out populations in villages, cities and even entire regions. From the dawn of humanity to modern times, every infectious disease outbreak has been accompanied by widespread fear among the masses. This is especially true for *Vibrio* pandemics, which have had a profound effect on human populations, including their evolution and cultural development. For instance, people were terrified when cases of cholera were reported, as it could arrive suddenly and spread rapidly through the population with horrific symptoms including massive rice-water diarrhea, bleeding fluid from the mouth and violent muscle contractions that continued even after death, which could occur within hours or days. However, this genus does not only include pathogens, but also encompasses species with complex mutualistic relationship, wide range of metabolic capabilities, inhabitants of deep seas and many others, demonstrating the huge variety within this genus. From the identification of *V. cholerae* as the causative agent of cholera and its route of transmission, to the first description of quorum sensing, the discovery of viable but nonculturable state and its designation as barometer of climate change, the genus has substantially impacted our understanding of microbiology. The intriguing history of *Vibrio* reveals remarkably scientific discoveries including examples that are still remembered today. The timeline of this genus, including the principal discoveries and milestones, is summarized here to showcase the profound impact *Vibrio* species have had on both science and society.

Introduction:

The genus *Vibrio* is a large group of aquatic bacteria, with nearly 150 individual species currently identified, including major human and animal pathogens (Ceccarelli et al., 2019). The term "*Vibrio*" is derived from the Latin word "*vibrare*", which means to vibrate or move rapidly (Dorland 2012) and it is considered one of the earliest names for a bacterial taxon. The common ancestor of all *Vibrio* species is estimated to have existed around 600 million years ago (Sawabe et al., 2007). During this long period, the lineage has undergone profound genetic changes and adaptations, allowing its members to evolve in a variety of environments, from marine and freshwater ecosystems to the guts of animals and even plants (Baker-Austin et al., 2018). Historically, much of the research and literature on *Vibrio* originated in the field of medical microbiology, focusing primarily on *V. cholerae*, the causative agent of cholera. Whereas research on *Vibrio* species commonly found in aquatic ecosystems has been conducted by environmental microbiologists, particularly those exploring pathogenic bacteria causing infections in marine animal hosts (Farmer and Hickman-Brenner, 2006). In both cases, important findings were identified, such as viable but nonculturable (VBNC) state and quorum sensing mechanisms. Considering both human and animals diseases, nowadays cholera often hits developing countries having poor sanitation, lack of hygiene and crowded living conditions (Baker-Austin et al., 2018). Meanwhile, in the aquaculture sector, the economic burden of *Vibrio* infections as aquatic animal pathogens is widespread and estimated at hundreds of billions of dollars worldwide. Notability, some *Vibrio* species have evolved into specialized pathogens, causing

diseases and even pandemics. In contrast, others have developed complex symbiotic relationships with their hosts. This mini-review summarizes the early breakthrough discoveries in *Vibrio* history, one of the most enigmatic bacterial genus that has substantially marked history and had a deep impact on human life (R. R. Colwell 1996, 2014).

The history of *Vibrio* genus: cholerae and *Vibrio cholerae*

Cholera caused by the bacterium *Vibrio cholerae* has been a major human scourge for centuries and has resulted in seven pandemics since 1817, including the seventh and ongoing pandemic, which is currently posing a health threat to at least 175 countries in the world (Balasubramanian et al. 2023). The etymology of the term "cholera" has been debated for many years, but it may provide hints to understanding the disease (R. R. Colwell 1996). Cholera may have been derived from the Greek words, chole (bile) and rein (flow), meaning the flow of bile (Lacey, 1995). Other researchers suggest that the name derives from the Greek word cholera, meaning gutter (Barua and Greenough 1991). The symptoms of cholera may have reminded the Greeks of the strong flow of water on gutters during a downpour. To distinguish the generic term cholera (gutter), from the disease cholera, the word "nousos" or disease was added to the latter (Lacey, 1995).

Up to 3rd century: The first historical records mentioning *Vibrio* predominantly refer to *V. cholerae,* the causative agent of cholera*,* which has been responsible for sporadic and devastating outbreaks affecting populations worldwide throughout history. The earliest disease descriptions can be found in the writings of Hippocrates (460-377 BC), Galen (129-216 AD), and Wang Shuhe (180-270 AD) (Macnamara, 1876).

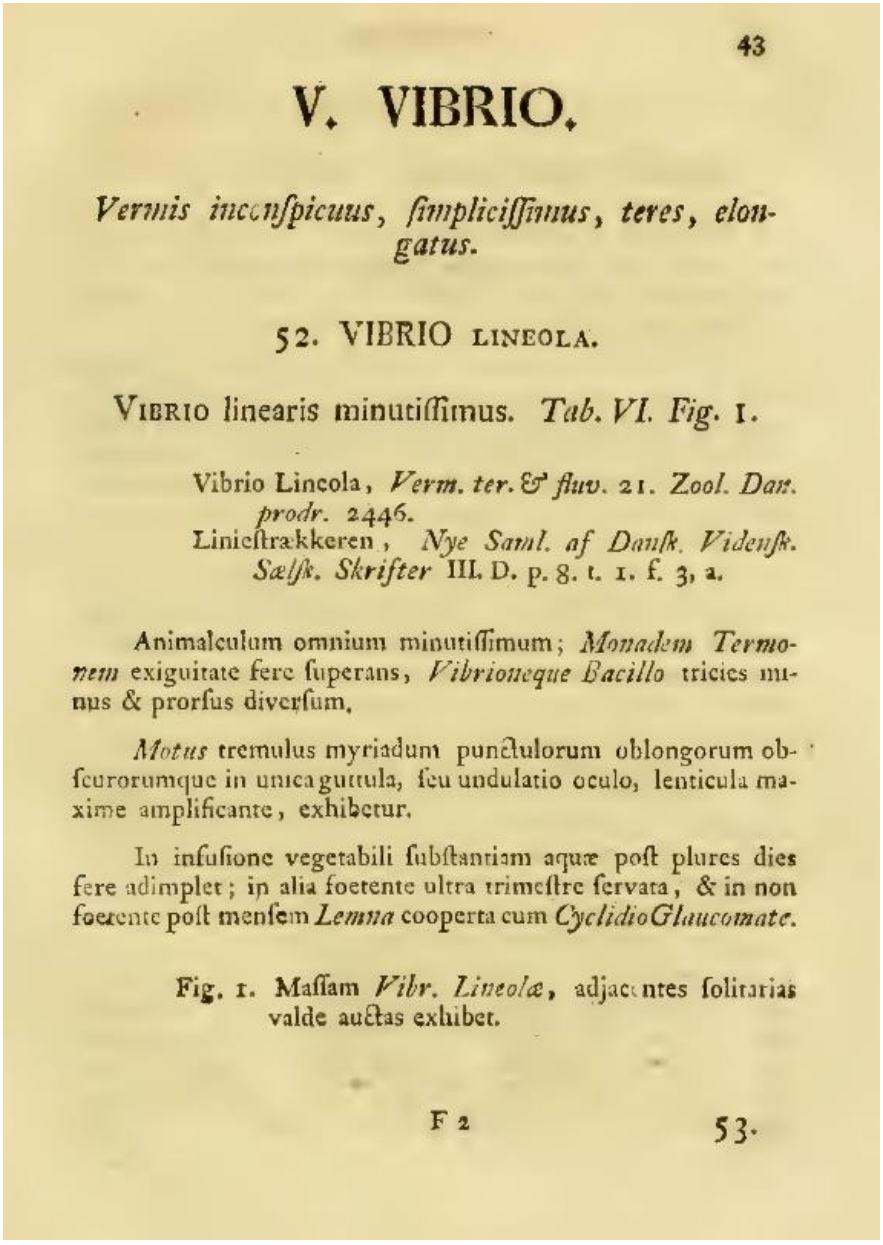
Several ancient Indian evidences also described a disease similar to cholera (Pollitzer, 1954). For instance, there was in a temple at Gujrat in western India, a monolith dating back to the time of Alexander the Great with the following inscription referring apparently to cholera:

> "The lips blue, the face haggard, the eyes hollow, the stomach sunk in, the limbs contracted and crumpled as if by fire, those are the signs of the great illness which, invoked by a malediction of the priests, comes down to slay the braves ... "

However, there is no evidence that these early records referred specifically to infections caused by *V. cholerae*, nor is it clear whether the disease manifested in the same epidemic way as is known today.

15th century: The first records of the existence of cholera date back to the 15th century in India, after the arrival of Portuguese explorer Vasco da Gama in 1498. In 1503, an epidemic of Asiatic cholera was reported in the army of Calicut, where about 20000 men died from a disease that struck them suddenly in the abdomen, some dying within 8 hours. This was followed by another outbreak in the population in 1543. The first documented reference to a cholera outbreak outside of India dates to the year 1629 and occurred in Jakarta (Macnamara, 1876).

18th century: The first description of the genus *Vibrio* was made by Müller in 1786, in his book *Animalcula Infusoria; Fluvia Tilia et Marina*. Müller included 15 species in this genus, providing names and descriptions for each. However, almost all the species originally placed in this genus were reclassified into other genera. For instance, species such as *V. rugula, V. undula, V. serpens*, and *V. spirillum* were reclassified by later researchers and placed in the genus *Spirillum*.



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V. VIBRIO.

Vermis inconſpicuus, ſimpliciſſimus, teres, elongatus.

52. VIBRIO LINEOLA.

VIBRIO linearis minutiſſimus. *Tab. VI. Fig.* 1.

Vibrio Lineola, *Verm. ter. & fluv.* 21. *Zool. Dan. prodr.* 2446.
Linieſtrækkeren, *Nye Saml. af Danſk. Videnſk. Sælſk. Skrifter* III. D. p. 8. t. 1. f. 3, a.

Animalculum omnium minutiſſimum; *Monadem Termonem* exiguitate fere ſuperans, *Vibrioneque Bacillo* tricies minus & prorſus diverſum.

Motus tremulus myriadum punctulorum oblongorum obſcurorumque in unica guttula, ſeu undulatio oculo, lenticula maxime amplificante, exhibetur.

In infuſione vegetabili ſubſtantiam aquæ poſt plures dies fere adimplet; in alia foetente ultra trimeſtre ſervata, & in non foetente poſt menſem *Lemna* cooperta cum *Cyclidio Glaucomate*.

Fig. 1. Maſſam *Vibr. Lineolæ*, adjacentes ſolitarias valde auctas exhibet.

F 2 53.

Figure 1 One of the first written descriptions of the genus Vibrio. The page was obtained from the book Animalcula Infusoria; Fluvia Tilia et Marina written in Latin by *Müller in 1786. Müller included 15 species in this genus, providing names and descriptions for each species. The book is available online at: https://archive.org/details/animalculainfuso00ml/.*

19th century: In 1838, Ehrenberg classified *Vibrio* organisms that were later recognized as *Schizomycetes*. Fuchs in 1841 described *V. cyanogenus* and *V. xanthogenus*, which were later identified as *Pseudomonas syncyanea* and *P. synxantha*, respectively. Many of the bacterial species initially included in the genus *Vibrio* before 1854 have now been reclassified to other genera (Hugh, 1964).

The significant turning point in the history of the genus *Vibrio* occurred during the third cholera pandemic (1846–1860), widely recognized as the deadliest in history. Originating in India, this pandemic caused a large number of victims across Asia, Europe, Africa, and North America. In 1849, John Snow became famous for tracing a cholera outbreak to a single contaminated well (Snow,

1855). This discovery was a milestone in the field of epidemiology and is still considered a canonical example today.

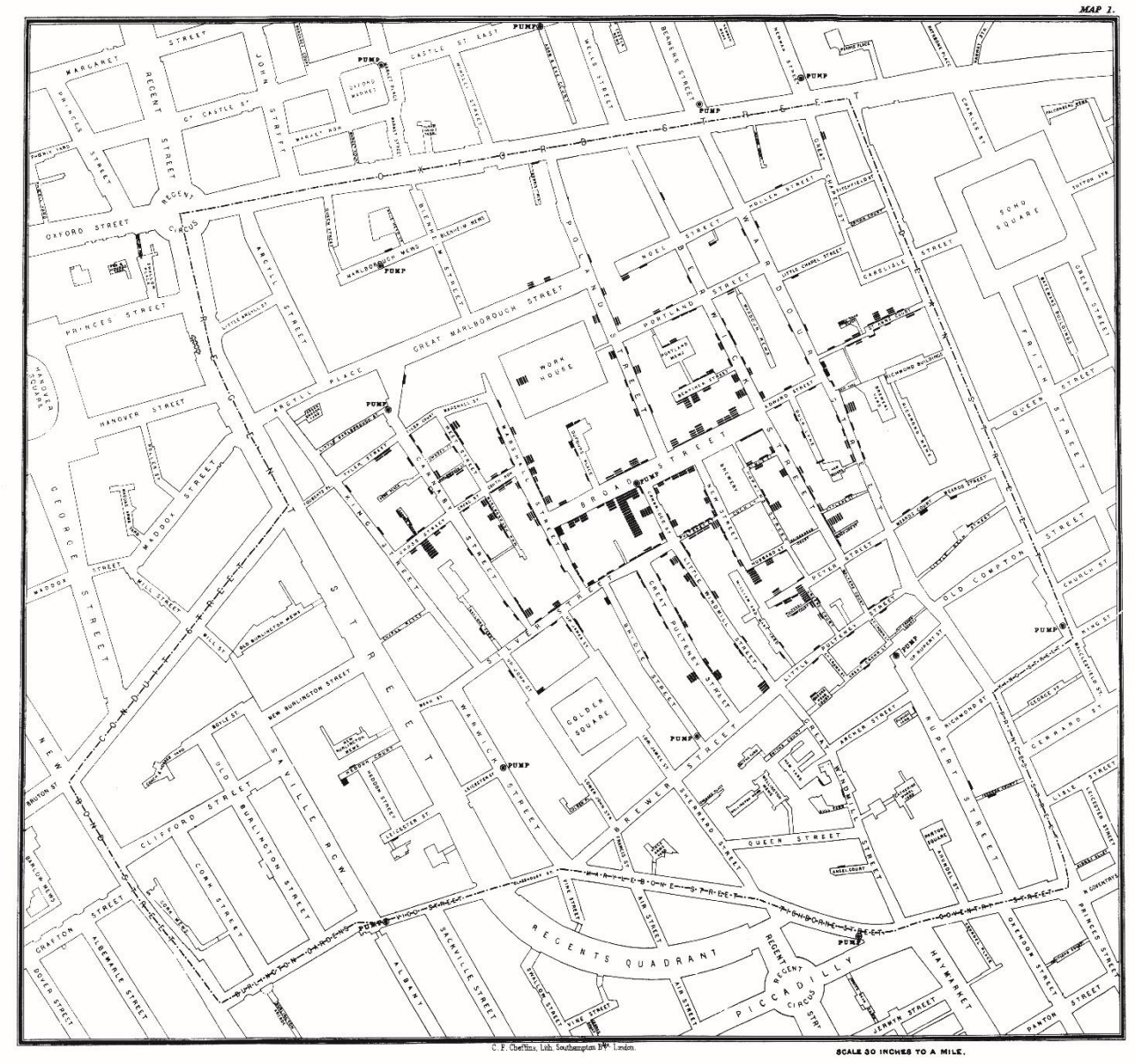

Figure 2 The map made by John Snow shown on December 4 1854, at a meeting of the London Epidemiological Society and then published in his book, On the Mode of Communication of Cholera. The map shows the deaths from cholera in Broad Street, Golden Square and the neighborhood from 19 August to 30 September 1854. Cholera cases are indicated as black stacked rectangles, while the famous pump is indicated as a black dot.

Snow work demonstrated that contaminated water was the source of the outbreak by tracing back the families of cholera patients living in the Soho district of London, leading him to conclude that contaminated water from the Broad Street pump was the source of the disease. As a result, the removal of the pump handle led to the end of the epidemic. Snow correctly identified the fecal-oral route as the transmission mode of human infection (Snow, 1855), contributing significantly to public health. Despite his efforts, Snow failed to identify the specific pathogen responsible for causing cholera. Thus, the predominant belief that cholera was an airborne disease persisted in decades to come (Lippi and Gotuzzo, 2014).

1854 was a particularly devastating year during the pandemic and marked a turning point in the history of the genus *Vibrio*. In that year, Filippo Pacini, a researcher in Florence, discovered that a bacterium was the cause of cholera. Utilizing microscopy during autopsies of cholera victims, Pacini observed a close association between the rupture of the intestinal lining and millions of bacteria, which he named *Vibrio cholerae*. Using his histological techniques, Pacini determined that the intestinal mucosa dysfunction led to debilitating symptoms such as diarrhea, vomiting, severe dehydration and death (Pacini, 1854). Unfortunately, his discovery was not recognized during his lifetime. Indeed, was not until 82 years after his death, when the international committee on nomenclature in 1965 adopted *Vibrio cholerae Pacini 1854* as the correct name of the cholera-causing organism.

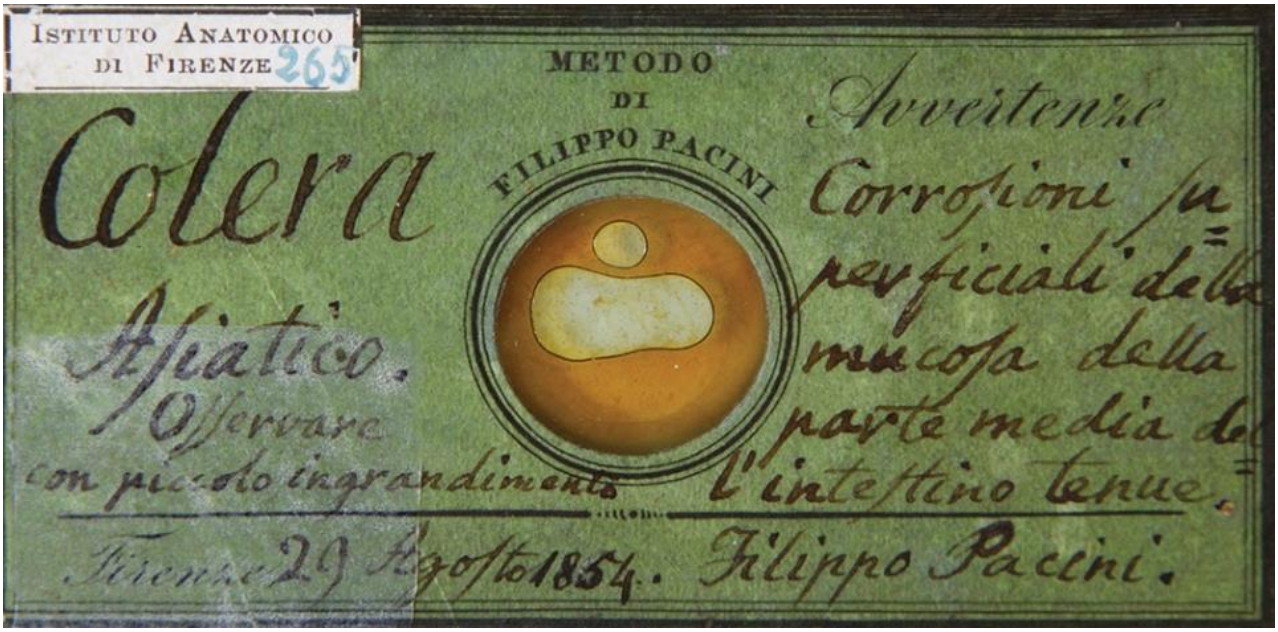

Figure 3 Microscope slide prepared by Pacini the 29th august 1854 containing V. cholerae.

In the same year and during the same period, towards the end of August, while in Barcelona there was a peak in cholera cases, Spanish pharmacist Joaquín Balcells y Pascual placed an open glass of pure water near a cholera patient, exposing it to the patient exhalations for three days. Although the water remained visually clear, a whitish deposit had formed at the bottom of the glass. Using his microscope, the pharmacist observed numerous vibrions exhibiting remarkable mobility, characterized by their angular movements (El Restaurador Farmacéutico n°30, 1854). Simultaneously, in Great Britain, the Report of the Committee for Scientific Inquiries, in relation to the cholera epidemic of 1854 was being compiled by the General Board of Health of the Medical Council and the Committee for Scientific Inquiries. In the report, it was claimed the presence of vibrios in the air of the cholera ward filled with patients. However, the report concluded that it was premature to infer a connection between the disease and these organisms, just because they were widespread (Report of the Committee for Scientific Inquiries in Relation to the Cholera Epidemic of 1854, 1857) irrespectively to the fact that cholera airborne transmission has never been proven.

However, it was not until 1884 that Robert Koch was able to isolate the causative agent of Asiatic cholera. Koch provided a detailed description of the organism, improving the understanding of *Vibrio cholerae* and its role in causing cholera (Koch, 1884). This discovery had a huge impact in the

understanding of infectious diseases and public health, resulting in the development of specific preventions and treatments for cholera. Indeed, in 1885, the Catalan bacteriologist Jaime Ferran y Clua formulated for the first time the cholera vaccine, based on live bacteria (Ferran J., 1885).

20th century: Almost a century later, the VBNC (Viable But Non-Culturable) state was discovered in *V. cholerae* (Xu *et al.*, 1982) adding another layer to the complexity of understanding this pathogen. VBNC is a unique survival strategy of bacteria in the environment in response to harsh environmental conditions. VBNC bacteria cannot be cultured on traditional microbiological media, but they remain viable and once the environmental conditions return favorable, they resuscitate and can even result virulent. The VBNC phenomenon may explain the sporadic nature of cholera outbreaks and the inability to recover *V. cholerae* from natural waters between epidemics. These results had significant implications for understanding the pathogenesis of this bacteria and for the study of microorganism physiology.

21th century: In recent times, the explosion in genomics and sequencing techniques, as well as the implementation of advanced microscopic, biochemical and molecular approaches have enabled a significant breakthrough in the understanding of the biology, ecology and epidemiology of Vibrio cholerae and the cholera infection (Colwell 1996).

Notably, the complete genome sequence of *Vibrio cholerae* El Tor N16961 revealed in 2000 provided insights into the environmental and pathobiological characteristics of this microorganism. A relevant finding was that *Vibrio cholerae* possessed two chromosomes, a genomic peculiarity shared among the *Vibrio* genus (Heidelberg *et al.*, 2000).

The history of *Vibrio* genus: Non-cholerae *Vibrio* species

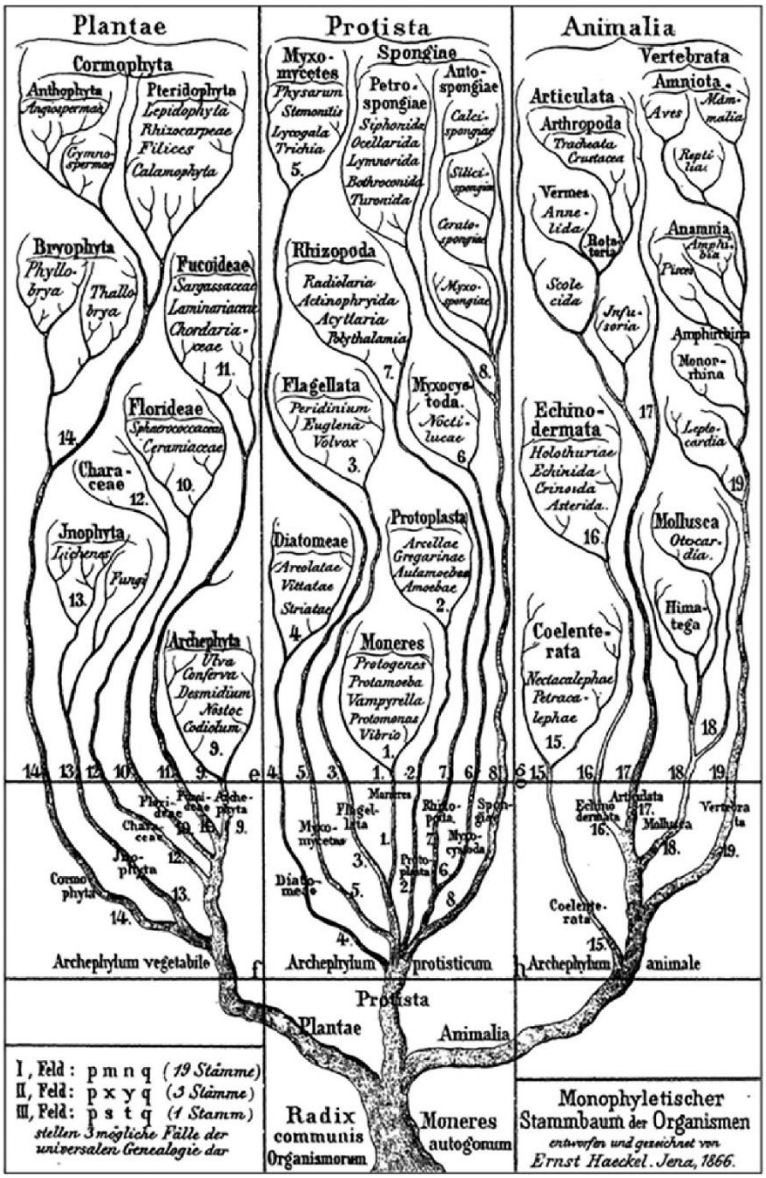

Figure 4 The first three-kingdom tree of life that included microorganisms was depicted by Ernst Haeckel in his book: General Morphology of Organisms 1866. Monera included microorganisms, such as Vibrio, that are today known as Bacteria, a term not yet coined in 1866.

18th century: To understand the importance of the *Vibrio* genus, we can refer to Ernst Haeckel tree, published in his *General Morphology of Organisms* (Haeckel, 1866) and considered the earliest "tree of life" model of biodiversity. Indeed, *Vibrio* is present in this first three-kingdom tree of life. Haeckel described *Vibrio* as "simple primordial cell masses, without a nucleus and without a shell or membrane, which remain at this lowest level of individuality throughout their lives".

Vibrio genus has also attracted the attention of environmental marine microbiologists, because the bacterial populations cultured from near-shore waters and those associated with fish and shellfish were predominantly *Vibrio*. The first non-pathogenic *Vibrio* species isolated from the aquatic environment, namely *V. fischeri* and *V. splendidus* were described by a Dutch microbiologist, Martinus Beijerinck in the late 1880 (Beijerinck, 1889; Robertson *et al.*, 2011; Thompson *et al.*, 2016).

19th century: In 1893, Canestrini reported epizootics in eels (*Anguilla vulgaris*) associated with a bacterium initially described as *Bacillus anguillarum* and later reclassified as *V. anguillarum*. *This bacterium* has historically been recognized as a major pathogen affecting marine animals (Smith, 1961). Another example is a disease observed in young Pacific salmon held in sea-water ponds (Rucker *et al.*, 1954). The disease, attributed to *V. anguillarum*, caused extensive hemorrhages in the muscles and internal organs of the fish. This disease typically occurred from April or May, when temperatures increased, and persisted throughout the summer.

20th century: *V. harveyi* is a halophilic *Vibrio* species first described as *Achromobacter harveyi* by Johnson and Shunk in 1936, who named it in honor of E. N. Harvey, a pioneer in the study of bioluminescence. It has been classified in *Lucibacterium* and *Beneckea* genera but is now included in the *Vibrio*. Many strains are bioluminescent, but also non-luminescent exist. *V. harveyi* has been isolated from various geographical locations and from coastal and open ocean seawater, as well as from the surfaces and feces of fish and squids (Baumann, 1981). Numerous papers have been published on its physiology and metabolism and it is often used in bioluminescence studies as model (Farmer and Hickman-Brenner, 2006). Mariners, on many occasions over the centuries, have reported observing "milky seas", a mysterious phenomenon where the ocean shines intensely and uniformly at night. This light emission appears to be due to luminous *Vibrio harveyi* living in association with colonies of the microalga *Phaeocystis*. However, the details of the formation mechanisms, spatial extent, global distribution, temporal variability and ecological implications of milky seas remain almost entirely unknown (Miller *et al.*, 2005).

On October 20 and 21, 1950, an outbreak of food poisoning due to the consumption of "Shirasu" (whitebait) occurred in the cities of Osaka, Kishiwada, Kaizaka and Izumi-Sano in *Japan. In this outbreak, 272 individuals developed symptoms of acute gastroenteritis, and 20 died. Initially, authorities suspected the outbreak to be a criminal case of poisoning. However, subsequent research led by Fujino at the Research Institute for Microbial Diseases (RIMD) of Osaka University succeeded to isolate Pasteurella parahaemolytica,* from *shirasu* samples and post-mortem material*. In the following years it was reclassified as Vibrio parahaemolyticus (T. Fujino et al., 1953; Fujino et al., 1965). In 1965, Zen-Yoji et al., demonstrated that many summer outbreaks of food poisoning in Tokyo were caused by the consumption of sushi contaminated with V. parahaemolyticus biotype 1. Subsequently, it was discovered that V. parahaemolyticus had an annual cycle of abundance in marine waters and estuaries depending on temperatures and living in association with zooplankton (Kaneko and Colwell, 1973, 1975). After the emergence of the pandemic O3:K6 strain in 1995-96 (Nair et al., 2007), this pathogen became one of the most globally significant marine pathogenic bacteria. Since then it has been responsible for epidemics on many other continents (Jaime Martinez-Urtaza et al., 2005; Yu et al., 2013).*

Nowadays, V. parahaemolyticus is the main cause of acute gastroenteritis in humans, caused by the consumption of raw, undercooked, or improperly processed seafood. *V. parahaemolyticus pandemic strains ST3 and ST36 together with V. cholerae O1 are the only known examples of marine bacterial pathogens capable of transcontinental expansion (Martinez-Urtaza et al., 2017). In 1968, R Sakazaki, proposed the new species name V. alginolyticus for biotype 2 of Vibrio parahaemolyticus, which can be pathogenic to both humans and marine animals.*

V. natriegens was first described as *Pseudomonas natriegens* by Payne in 1961 who isolated it from salt marsh mud on Sapelo Island off the coast of Georgia, USA. *V. natriegens* utilizes a wider variety

of carbon sources than other marine vibrios, making it easy to isolate and identify. It also has the shortest generation time (9.8 min) of any bacterium (Eagon, 1962), making it a popular species for use in scientific educational exercises and physiological studies. Since then, *V. natriegens* has been isolated from coastal seawater in several locations (Farmer and Hickman-Brenner, 2006). *Vibrio natriegens* has been considered a valid, faster-growing alternative to *E. coli* to utilize as a model organism for molecular biology (Weinstock *et al.*, 2016).

V. campbellii is a halophilic species first described by Baumann *et al.*, in 1971, who isolated 60 strains from ocean water off the coast of Hawaii. They noted considerable variation in the phenotypic properties of this large group but included all of them in *V. campbellii*. Grimes *et al.*, in 1986 isolated 20 strains from pelagic water during a voyage from Barbados to Puerto Rico to Bermuda and concluded that *V. campbellii* is also found in the open waters of the Atlantic Ocean. This finding established that *Vibrio* does not only inhabit coastal waters but also has an oceanic lifestyle.

V. vulnificus was first reported in 1976 by Hollis *et al.*, in the USA. Unlike other *Vibrio* species, *V. vulnificus* caused extraintestinal infections in humans and showed biochemical characteristics that distinguished it from other species. Initially referred to as L+ (lactose positive) *Vibrio*, in 1979 it was observed L+ *Vibrio* could cause infection, particularly during the summer (Blake *et al.*, 1979). *V. vulnificus* is relatively rare but extremely dangerous, with a high mortality rate of about 50%. It can cause necrotizing fasciitis, commonly known as flesh-eating bacteria syndrome, a rapid and severe infection that destroys skin, fat, and muscle tissue. It can also lead to primary septicemia, a life-threatening infection of bloodstream, especially in individuals with compromised immune systems or underlying chronic conditions such as liver disease. In late 1979, J. J. Farmer proposed the name *Vibrio vulnificus* (vulni = wound, ficus = to make) for this distinct pathogen due to its association with a specific type of skin lesion.

V. mimicus, first observed around 1980, emerged as a distinct species due to unusual biochemical traits in *V. cholerae* strains. These distinctive traits were labeled as V. cholerae—lysine-decarboxylase negative, *V. cholerae*—mannitol negative, or *V. cholerae*—sucrose negative. Through DNA hybridization and phenotypic analysis, researchers found that five out of the six unique biogroups were closely related to *V. cholerae* (Davis *et al.*, 1981). These were correctly identified as atypical strains of *V. cholerae*. However, a group of sucrose-negative strains displayed significant divergence and distinct phenotypic characteristics compared to *V. cholerae*, leading the researchers to propose a new species named *Vibrio mimicus*. The name 'mimicus' refers to the fact that these strains mimic *V. cholerae* (Davis *et al.*, 1981).

Grimes *et al.*, in 1984 studied two urease-positive halophilic vibrios isolated from a brown shark (*Carcharhinus plumbeus*) that had died in captivity in a large aquarium. One strain was identified as *V. damsela*, a *Vibrio* species known to cause human wound infections and found in the marine environment, causing skin lesions on certain marine fish, first described by Love *et al.*, 1981. The other strain could not be identified. After further phenotypic testing and DNA-DNA hybridization, Grimes et al. 1984 concluded that this was a new species and named it *V. carchariae*. Subsequent to the original report, V. carchariae has also been isolated from other sharks but has also been shown to be present in human clinical specimens. Pavia *et al.*, 1989 described a case where this species was isolated from a wound after a shark bite. An 11-year-old girl was attacked by a shark on the coast of South Carolina. She suffered several deep lacerations to her left calf, which became infected after subsequent surgery. A culture of the infected wound yielded an unusual *Vibrio* that

was later identified as *V. carchariae*. However, taxonomic evidence that *Vibrio carchariae* is a junior synonym of *Vibrio harveyi* (Pedersen et al., 1998).

In January 1888, Martinus Beijerinck received a piece of salt pork that glowed in the dark from Mr. Enklaar of Deventer. Beijerinck lab journal entry from January 12 notes that the flesh of the pork produced light and that some areas were brighter than others. He observed a mixture of bacteria, predominantly diplococci. Despite efforts to isolate the light-producing species from the pork, his attempts were unsuccessful, possibly due to the absence of sodium chloride in his medium, which consisted of pork, gelatine, peptone, and sodium carbonate. Nevertheless, his curiosity persisted. On January 16, Beijerinck placed a piece of plaice fish on an open plate in his cellar. By January 22, the fish was glowing, enabling him to isolate the light-producing bacteria using a medium based on fish and seawater (Robertson *et al.*, 2011).

However, the process behind bioluminescence, which now it is known to be the quorum sensing, was still not known. Prior to 1994, quorum sensing was commonly referred to as "autoinduction" (Fuqua et al., 1994; Nealson et al., 1970). Autoinduction was originally described for *V. fischeri* in the early 1970s. The experiments conducted by Kempner and Hanson (1968) revealed the induction of bioluminescence in freshly inoculated *V. fischeri*. The culture emitted luminescence in response to medium previously conditioned with the same strain. Nealson et al. (1970) were the first to propose that autoinduction of luminescence in *V. fischeri* occurs at the transcriptional level and that the process is regulated by extracellularly secreted components (Eberhard, 1972). The term “quorum sensing” was introduced by Steven Winans in 1994, who wrote one of the first review on autoinduction in bacteria (Turovskiy et al., 2007). Furthermore, one of the most incredible and elegant forms of interaction and coevolution of *Vibrio* with aquatic organisms is the one discovered by McFall-Ngai and Ruby in 1991, a mutualistic symbiosis between the Hawaiian squid (Euprymna scolopes) and the luminous *V. fischeri*, which colonizes the light organ of the squid. This was not the first discovery of a symbiotic relationship between *V. fischeri* and marine animals (Nealson, 1976), but it is considered the most iconic and fascinating one. This complex symbiosis involves colonization, quorum-sensing-induced bioluminescence, diurnal rhythm, maintenance and dispersal, highlighting the intriguing interaction between *Vibrio fischeri* and the Hawaiian squid. For these important discoveries, *V. fischeri* has become a model and one of the most studied microorganisms for studies on bioluminescence, quorum sensing, cell density-dependent behavior and the mechanisms underlying beneficial host–microbe interactions. It is also used in the Microtox bioassay to detect toxic substances in different substrates, where exposure to a toxic substance causes disruption of the respiratory process of the bacteria, resulting in reduced light emission.

In 2002, a novel temperature-dependent pathogen causing bleaching and lysis to the coral *Pocillopora damicornis* was isolated in Zanzibar. Most coral diseases causative agents remain unknown, but this particular strain caused rapid destruction of coral tissue within two weeks at water temperatures above 26°C. It was classified as a member of the *Vibrio* genus and received the species name “*coralliilyticus”* due to its deadly activity against corals (Ben-Haim and Rosenberg, 2002).

The availability of new resources and the release of chemical substances in the oceans have likely influenced the selection pressures also on *Vibrio* populations, leading to the emergence of new lineages and adaptations. An example is *Vibrio cyclitrophicus*, which was isolated from creosote-contaminated marine sediments in 2001. This strain demonstrated the ability to utilize various polycyclic aromatic hydrocarbons as carbon substrates, including naphthalene, 2-methylnaphthalene, and phenanthrene (Hedlund and Staley, 2001).

V. diazotrophicus, a nitrogen-fixing *Vibrio*, has been isolated from diverse sources, such as the gastrointestinal tracts of sea urchins collected in Nova Scotia, Canada and the surfaces of reeds growing in a drainage ditch in Kent, England. (Guerinot *et al.*, 1982). The expanding role of vibrios in the environment, particularly in nutrient cycling, has begun to be recognized (Colwell, 2014).

Horizontal gene transfer, the process of genetic material exchange between bacteria, has been identified as a key factor in the evolution and adaptation of *Vibrio*. For instance, *V. vulnificus* biotype 3 is thought to have evolved from biotype 1 through the acquisition of unique genes from other bacterial species, such as *Shewanella*, which occupy the same ecological niche (Efimov *et al.*, 2013). Another significant case is *Vibrio parahaemolyticus*, which emerged in 1995 and caused a pandemic. Analyses suggest that the initial non-pathogenic founder clone of the O3:K6 type acquired through horizontal gene transfer a new toxRS region and at least seven novel genomic islands. Whole-genome sequencing and comparisons have confirmed these observations and highlighted the role of gene conversion involving horizontally transmitted DNA in these genetic changes, as described in various studies (Espejo *et al.*, 2017).

Vibrios are undoubtedly relevant inhabitants of riverine, estuarine, and marine aquatic environments, including the deep sea (Bosi *et al.*, 2024). Notably, *V. antiquarius* (Hasan *et al.*, 2015) and *V. bathopelagicus* (Lasa *et al.*, 2021), which could be related to coastal pathogenic strains, have genomes that encode genes involved in both adaptation to the deep sea and virulence. This suggests that the evolution of virulence traits as adaptations to environmental niches outside the host is likely a common feature among marine microbial pathogens (Lasa *et al.*, 2021).

Vibrio is considered an indicator of climate change due to its sensitivity to warm and low-salinity waters. Research shows that as marine environments warm, there is a significant increase in *Vibrio* infections in humans and aquatic animals. There is also evidence of its positive relation with extreme weather events like heatwaves (Doni *et al.*, 2023). This correlation is more evident in temperate regions, such as Northern Europe, where outbreaks have become more frequent with rising sea temperatures (Vezzulli *et al.*, 2016). Projections indicate that, by 2100, under the least favorable climate scenario, coastal areas suitable for *Vibrio* could extend by 38,000 km, with an increase in seasonal suitability of approximately one month every 30 years (Trinanes and Martinez-urtaza, 2021). This highlights the importance of understanding and monitoring *Vibrio* as a "microbial barometer" of climate change, highlighting the need for integrated approaches that combine microbiology, genomics, epidemiology and climate science to address the emerging threat of *Vibrio*-associated diseases (Baker-Austin *et al.*, 2017).

Conclusion:

As we continue to unravel *Vibrio* evolution, scientists are gaining an enhanced understanding of the genetic and ecological factors that have shaped this diverse group of bacteria. Such knowledge is crucial for developing strategies to combat *Vibrio*-related diseases, mitigate their impact on marine ecosystems, and anticipate potential future challenges in a changing world. Remarkably, vibrios represent a group of microorganisms that have been continuously studied for almost two centuries.

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B) The big four: *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus* and *V. vulnificus*

Introduction:

The *Vibrio* genus consists of 151 confirmed species (http://www.bacterio.net/vibrio.html). Among them, around twelve species have been identified as capable of causing infections in humans (Ceccarelli *et al.*, 2019) *Vibrio* spp. are a prevalent group of Gram-negative, rod-shaped bacteria that naturally inhabit freshwater, estuarine, and marine environments. These bacteria share various biological and genomic characteristics. Their genetic material is distributed between two chromosomes, which have been influenced by recombination and horizontal gene transfer and their abundance in the natural environment tends to mirror environmental temperatures (Baker-Austin *et al.*, 2018).

V. cholerae, the causative agent of cholera, and *V. vulnificus*, the deadliest seafood-borne pathogen, are both notorious for their severe impact on human health. *V. parahaemolyticus* is renowned for causing foodborne gastroenteritis from raw seafood consumption, while *V. alginolyticus* gained fame as a marine bacterium associated with wound infections and occasional food poisoning, especially in warm coastal regions (Takemura *et al.*, 2014).

Several reports have recently indicated that human *Vibrio* illnesses are increasing worldwide, as well as the species responsible for these infections. Human infections can be acquired from more than one route of exposure (e.g., consumption of seafood and exposure to contaminated water). Globally, these four pathogens disproportionately dominate human infection reports associated with vibrios, although it should be noted that also other *Vibrio* species, including *V. damsela*, *V. hollisae*, *V. mimicus* and *V. metshnikovii*, have also been implicated in human infections (Baker-Austin *et al.*, 2018).

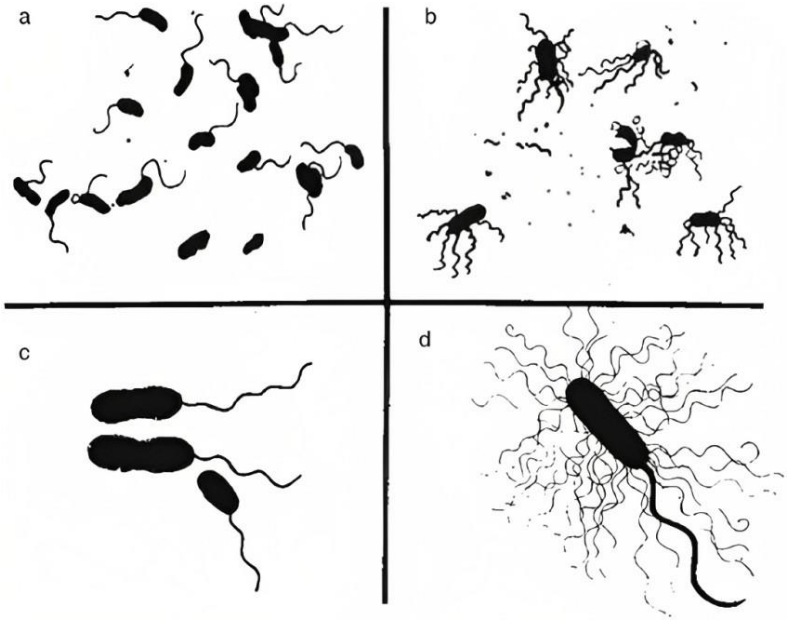

Figure 5 Cellular morphology of Vibrio cholerae, V. parahaemolyticus, and V. alginolyticus. (a) V. cholerae from an 18-h culture on nutrient agar (Van Ermengen cilia stain). (b) Flagella stain of V. parahaemolyticus. (c) Electron micrograph of V. parahaemolyticus; note single-sheathed polar flagellum. (d) Electron micrograph of V. alginolyticus grown on solid media; peritrichous flagella are

present; note their different size and shape as com- pared to the sheathed polar flagellum. (From Farmer et al., 1985)

V. CHOLERAE

Once regarded as one of the world's most feared bacterial pathogens (Pollitzer, 1954), *Vibrio cholerae* is a curved rod-shaped bacterium that does not form spores and is naturally found in brackish and estuarine environments worldwide. It is classified based on its major surface antigen, known as the O antigen, and can be divided into approximately 206 serogroups (Chowdhury *et al.*, 2017).

During the 19th century, cholera spread across the world from its original reservoir in the Ganges delta in India. Six subsequent pandemics killed millions of people across all continents. The current (seventh) pandemic started in South Asia in 1961, reached Africa in 1971 and the Americas in 1991. Cholera is now endemic in many countries. In 2017, WHO announced a global strategy aiming to end this pandemic by 2030 (https://www.who.int/news-room/fact-sheets/detail/cholera). Significant differentiations exist within this species based on the production of cholera enterotoxin (Ctx), serogroup classification, and the potential for epidemic transmission. Serogroups O1 and O139 have been specifically associated with epidemic cholera disease (J B Kaper, J G Morris and MM Levine 1995; Safa *et al.*, 2010). However, occasional strains belonging to serogroups other than O1 or O139 have been identified as pathogenic due to the production of Ctx or other virulence factors. It is worth noting that none of these alternative serogroups have caused large-scale epidemics or pandemics (Ceccarelli *et al.*, 2019). According to the World Health Organization (WHO), the global burden of cholera is estimated to be 1.3 to 4.0 million cases with 21,000 to 143,000 deaths every year (Ganesan *et al.*, 2020).

V. cholerae O1

V. cholerae O1, which produces cholera toxin (Ctx), is strongly associated with epidemic and pandemic cholera outbreaks (J B Kaper, J G Morris and MM Levine 1995). Ctx-negative *V. cholerae* O1 strains are generally considered non-pathogenic, although they have been isolated in some cases of diarrhea or extraintestinal infections. The O1 serogroup is further divided based on the presence of specific somatic antigens into two major serotypes, Inaba and Ogawa, as well as an unstable intermediate type called Hikojima (Baker-Austin *et al.*, 2017).

V. cholerae O1 can also be classified into two biotypes, classical and El Tor, which differ in various phenotypic and genotypic characteristics, including their pathogenic potential, survivability, and infection patterns in humans. Phenotypic tests such as sheep erythrocyte hemolysis, chicken cell agglutination, Voges-Proskauer, sensitivity to polymyxin B, and specific phage assays, along with genotypic analysis of specific genes (tcpA, rstR, and ctxB), are commonly employed to differentiate between the two biotypes . (Safa *et al.*, 2010).

However, both genetic and phenotypic diversity have emerged among circulating *V. cholerae* El Tor strains in Asia and Africa, giving rise to new pathogenic variants known as atypical El Tor variants (Goel *et al.*,2008). These variants include V. cholerae O1 hybrids that cannot be classified based on phenotypic tests and can produce cholera toxin of either biotype, as well as altered El Tor variants that produce cholera toxin of the classical biotype but can be classified as El Tor using conventional phenotypic assays (Grim *et al.*, 2010). These new variants have subsequently replaced the

prototypical seventh-pandemic *V. cholerae* O1 El Tor strains in terms of their frequency of isolation from clinical cholera cases (Ceccarelli *et al.*, 2019).

V. cholerae O139 Bengal

V. cholerae O139 Bengal emerged in the early 1990s when reports of a severe cholera-like epidemic surfaced in eastern India and Bangladesh (Albert, 1994). Further investigations identified the causative bacterium as belonging to a new serogroup called O139, also known as "Bengal," named after its place of origin. *V. cholerae* O139 can be distinguished from typical El Tor *V. cholerae* O1 strains based on clinical manifestations and the sequence of cholera toxin (Ctx). Unlike O1 strains, this bacterium does not produce the O1 lipopolysaccharide (LPS) due to the insertion of a 35-kb region encoding the O139 antigen (Dutta *et al.*, 2013) and it also produces a polysaccharide capsule.

Initially, there were concerns that the O139 serogroup would replace the O1 serogroup and lead to a new pandemic (the eighth pandemic) of cholera, especially in Southeast Asia. However, the number of reported O139 cases outside of Southeast Asia remained low, and O1 cases quickly regained dominance in the region (Albert and Nair, 2005; Ramamurthy *et al.*, 2022).

V. cholerae Non-O1 Non-O139

In recent years*, Vibrio cholerae* isolates that tested negative for the O1 serogroup have been referred to as *V. cholerae* non-O1 or non-agglutinating vibrios. Most of these strains do not produce cholera toxin (Ctx) and are not commonly associated with epidemic diarrhea (Vezzulli *et al.*, 2020). Since the emergence of the O139 serogroup, these strains are collectively known as *V. cholerae* non-O1/non-O139 (NOVC). While the majority of these strains do not produce Ctx, some strains may produce other toxins and are generally associated with self-limiting gastroenteritis or mild extraintestinal symptoms. However, there have been reports of fatal cases of necrotizing fasciitis and septicemia associated with NOVC (Vezzulli *et al.*, 2020).

Strains belonging to the O141 serogroup have been isolated from sporadic cases of severe diarrhea. These strains are known to produce Ctx and possess the toxin-coregulated pilus (Tcp) colonization factor typically found in O1 and O139 strains. Both toxigenic and nontoxigenic NOVC strains have caused several disease outbreaks in Asia and other countries in recent years. These outbreaks are believed to be related to ongoing climate change and ocean warming(Le Roux *et al.*, 2015; Baker-Austin *et al.*, 2017). Indeed, NOVC-related infections are on the rise and represent one of the most striking examples of emerging human diseases linked to climate change. NOVC strains are also believed to potentially contribute to the emergence of new pathogenic strains including strains with epidemic potential as a direct consequence of genetic exchange mechanisms such as horizontal gene transfer and genetic recombination(Vezzulli *et al.*, 2020).

V. PARAHAEMOLYTICUS

Vibrio parahaemolyticus is a significant cause of foodborne illness, particularly in Asia, South America, and the United States. It is recognized as the leading cause of human gastroenteritis associated with the consumption of seafood, especially raw fish and shellfish. The bacterium was first described in 1950 following a severe foodborne outbreak in Osaka, Japan, linked to the consumption of a small, semi-dried fish called "shirasu" (Fujino *et al.*, 1953). Since then, *V. parahaemolyticus* has been implicated in numerous diarrheal disease outbreaks worldwide.

According to data from the CDC FoodNet, the incidence rate of laboratory-confirmed *V. parahaemolyticus* cases was reported as 0.24 per 100,000 persons in 2015, making it the most frequently isolated *Vibrio* species from clinical specimens in the United States (accounting for 59.5% of infections attributed to members of the *Vibrionaceae*). However, considering underreporting and underdiagnosis, it is estimated that around 35,000 cases of foodborne *V. parahaemolyticus* infections occur each year in the United States (Gavilan *et al.*, 2023).

Vibrio parahaemolyticus possesses three antigenic components: H (flagellar), O (somatic), and K (capsular). The H antigen is common to all strains and is not used for serotyping. Instead, serotyping is based on the O and K antigens. The O antigen comprises 13 lipopolysaccharide antigens, designated O1 to O13, while the K antigen consists of 65 acidic polysaccharide antigens, named K1 to K71 (some antigens have been excluded). It is worth noting that many environmental and some clinical isolates cannot be typed for the K antigen (KUT), but most strains can be classified based on their O type. Until the mid-1990s, there appeared to be no correlation between serotype and virulence. However, the emergence of the pandemic strain O3:K6 in 1996 provided new insights into the importance of serotyping in understanding and tracking the spread of specific virulent strains during outbreaks.

V. parahaemolyticus serotype O3:K6

In 1996, a sudden increase in gastroenteritis cases attributed to *Vibrio parahaemolyticus* serotype O3:K6 was observed in Kolkata, India (Okuda *et al.*, 1997). Analysis of these O3:K6 isolates revealed a specific profile characterized by being tdh-positive, trh-negative, and urease-positive. Within a few months, strains with the same characteristics were isolated in several Southeast Asian countries, as well as in Japan and Korea (Nair *et al.*, 2007). Further analysis identified other distinctive molecular properties, including the presence of a filamentous phage (f237) with a unique open reading frame (orf8) and different sequences of the toxR and toxS genes (Matsumoto *et al.*, 2000). These molecular markers were subsequently used to develop specific PCR assays for the detection of the pandemic strain (group-specific PCR). Screening for this molecular profile, along with evidence obtained through RFLP-PFGE (restriction fragment length polymorphism-pulsed-field gel electrophoresis), facilitated the rapid identification of genetic patterns distinct from the epidemic O3:K6 clone in isolates belonging to different serotypes, such as O4:K68, O1:K25, O1:K41, O1:KUT, and O6:K18 (Chowdhury *et al.*, 2000). These clonal serotypes, exhibiting different antigenic formulas (with 27 different serotypes reported to date (Han *et al.*, 2017), are collectively referred to as O3:K6 serovariants, the O3:K6 pandemic clone, or the pandemic clonal complex. By the end of 2006, O3:K6 *V. parahaemolyticus* serovariants had been isolated in Europe, Africa, the United States, and several South and Central American countries (Nair *et al.*, 2007), marking the first pandemic of *V. parahaemolyticus*. Based on MLST analysis, sequence types (STs) can be attributed to clinical or environmental isolates. These STs can be further categorized into defined clonal complexes (CCs)

based on their phylogenetic relatedness. Currently, there are over 1,800 STs identified. For instance, the O3:K6 pandemic strains are described as ST3 and CC3. CC3 includes the O3:K6 serovariants, which are responsible for the pandemic outbreak. Another noteworthy strain is the O4:K12 strain of *V. parahaemolyticus*. This particular strain has been associated with repeated and diffuse outbreaks occurring on the Pacific and Atlantic coasts of the United States, as well as in Spain. It is reported as ST36 and CC36. ST3 was the only known example of *V. parahaemolyticus* transcontinental expansion until 2012, when the new type, ST36, was identified outside its endemic region, the Pacific Northwest of North America (Martinez-Urtaza *et al.*, 2017; Abanto *et al.*, 2020).

V. VULNIFICUS

Vibrio vulnificus is a pathogen that is responsible for the majority of seafood-associated deaths worldwide. This bacterium is classified as "accidental," meaning that it infects humans unintentionally. It can also be classified as opportunistic or secondary, as it typically causes severe disease only when the host's immune system is compromised or impaired.

Vibrio vulnificus was isolated from a series of lactose-positive and indole-positive vibrios obtained from both environmental and clinical sources in the United States (J. J. Farmer, 1979). Simultaneously, the isolation of indole-negative vibrios with similar characteristics from diseased farmed eels was reported in Japan. Subsequently, the indole-positive and indole-negative strains were assigned to two different biotypes (Tison *et al.*, 1982). Later, a third biotype was established in Israel to group atypical isolates (negative for cellobiose, salicin, and lactose fermentation) associated with human infection outbreaks linked to the handling of farmed tilapia (Bisharat *et al.*, 1999). All three biotypes can cause human diseases, but only biotype 2 has the additional ability to cause fish vibriosis. This ability relies on a plasmid called pVvBt2, which encodes a resistance system against the fishs's innate immune response (Amaro *et al.*, 2015). Over time, the diversity and number of isolates of the three biotypes have increased. Currently, there is no definitive test or set of tests to clearly differentiate among them, except for biotype 2, which can be identified by targeting unique plasmid genes through PCR. A recent phylogenomic analysis of the core genomes of 80 *V. vulnificus* strains, encompassing the three biotypes, revealed that the species can be subdivided into five well-supported phylogenetic groups or lineages. Interestingly, these lineages do not align with the biotypes previously established based on phenotypic and genetic characteristics (Roig *et al.*, 2018).

V. ALGINOLYTICUS

Vibrio alginolyticus is a bacterial species initially considered a biotype of *Vibrio parahaemolyticus* (R Sakazaki, 1968). Although genetically similar, these two species can be easily differentiated phenotypically, particularly through the fermentation of sucrose *by V. alginolyticus*. Sawabe *et al.*, 2013 has placed *V. alginolyticus* in the Harveyi clade of the genus *Vibrio*. This clade consists of several species found in seawater, saltmarsh mud, marine animals, and coral mucus. There are some differences in taxonomic traits between clinical and environmental isolates of V*. alginolyticus*, but the significance of these differences has yet to be fully demonstrated. Fortunately, there are various diagnostic methods available for the detection and discrimination of *V. alginolyticus* in different sample types. These methods include conventional, multiplex, and real-time PCR assays (Wei *et al.*, 2014). *Vibrio alginolyticus* is commonly found in seawater and seafood worldwide. It can be easily isolated from various marine organisms such as fish, clams, crabs, oysters, mussels, and shrimp, as well as from water samples (Baker-Austin *et al.*, 2018). Numerous studies conducted in different countries have consistently identified this species as one of the most prevalent vibrios in both

environmental and food samples. Moreover, researchers have observed a correlation between temperature and the rate of its isolation, with higher occurrences during warmer months (Tall *et al.*, 2013). In general, *V. alginolyticus*-related diseases primarily affect individuals who have had direct contact with seawater or handled shellfish. This bacterium is commonly associated with ear infections, including otitis media and otitis externa, as well as superficial wounds after exposure to contaminated water sources (Bultmann *et al.*, 2016). In certain cases, these infections can progress to more severe conditions like bacteremia and necrotizing fasciitis, especially in patients with compromised immune systems. Moreover, *V. alginolyticus* has been implicated as a food-associated pathogen. It has been detected and isolated from the watery diarrheal stools of patients with acute enterocolitis, as well as from the seafood they consumed. This highlights the potential risk of infection from consuming contaminated seafood (Baker-Austin *et al.*, 2018). Overall, *V. alginolyticus* poses a health concern for individuals exposed to seawater and seafood, and it is crucial to take proper precautions and hygiene measures when dealing with these environments and food sources to prevent infection.

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C) Ecological Complexity and Survival Strategies of *Vibrio* in the Aquatic World

Introduction:

All *Vibrio* species are aquatic heterotrophs living between the sediments and the water column. They can live freely or attached in aquatic environments, albeit their frequency varies with the aquatic ecosystem and niche. Numerous studies conducted by both culture -dependent and -independent methods indicated that the abundance of vibrios is high near or on the body of marine organisms, such as mollusks, fish, corals, and shrimp but especially plankton (Thompson *et al.*, 2016). The close association of *Vibrio* with plankton is considered a survival advantage (Erken *et al.*, 2015). For instance, *Vibrio cholerae* can form microcolonies on surfaces in as little as 15 minutes. It then produces exopolysaccharides that help stabilize the structure of its biofilm. Within 72 hours, a biofilm with a thickness of about 15 micrometers, comprising exopolysaccharides pillars and water channels, is formed (Thompson *et al.*, 2016). Recent advances in understanding *Vibrio* ecology have shifted the perspective from a simplistic model, where these bacteria interact with humans through waterborne transmission, to a far more intricate framework. This comprehensive view encompasses the effects of global weather patterns and climate change, ocean currents, diverse aquatic habitats, and interactions with phages and plankton. Additionally, it considers the adaptability of the *Vibrio* genome, along with the significance of rapid replication in their lifecycle. Together, these factors illustrate a complex network of interactions between this genus, their hosts, and the environment, highlighting the importance of biocomplexity in understanding *Vibrio* ecology (Thompson *et al.*, 2016).

Vibrio ecology

Vibrios exhibit a wide range of ecological adaptations, occupying diverse habitats ranging from free-living forms to those attached to biotic surfaces (e.g., copepods, crustaceans, fish, algae) and abiotic surfaces (Baker-Austin *et al.*, 2018). These diverse strategies contribute to their ecological success and persistence in marine ecosystems. Temperature and salinity have a are fundamental role in influencing the density and diversity of *Vibrio* species. Although they are eurythermic(5 ◦C to >40 ◦C) and worldwide distributed, they prefer warm waters (>18°C), and warmer periods and seasons, a trait that can explain their seasonal peaks. In temperate and cold climates, their density is higher in summer than in winter (Böer *et al.*, 2013). Despite the general relationship observed between the presence and abundance of *Vibrio* in higher water temperatures, species have specific responses to temperature: some prevail the whole year, while others were only detected at a specific temperature range or are less affected by this parameter. *V. alginolyticus* and *V. parahaemolyticus* were both found to be present even at temperatures around freezing point (Böer *et al.*, 2013). Of all the *Vibrio* spp., only a few are able to live in low salinity conditions: *V. cholerae, V. fluvialis, V. furnissii*, and *V. mimicus* (Sampaio *et al.*, 2022). Vibrios take advantage of the dynamics of natural food chains, especially during phytoplankton blooms, which release significant nutrients in both dissolved and particulate forms, sustaining high densities of Vibrios. The agglomeration of microalgae during blooms also provides extensive surfaces for *Vibrio* attachment, aiding their spreading and the diversification of their biological niches (Sampaio *et al.*, 2022). The abundance of phytoplankton leads to zooplankton blooms, particularly copepods that feed on phytoplankton, resulting in an

increase in *Vibrio* populations in nutrient-rich waters (Huq *et al.*, 1983; Alam *et al.*, 2006; Vezzulli, Pezzati, *et al.*, 2015; Brumfield *et al.*, 2021).

Remote imaging technologies developed by the US National Aeronautics and Space Administration have been used to relate chlorophyll A levels to cholera outbreaks, demonstrating a remarkable similarity between the two trends (Ford, 2009).

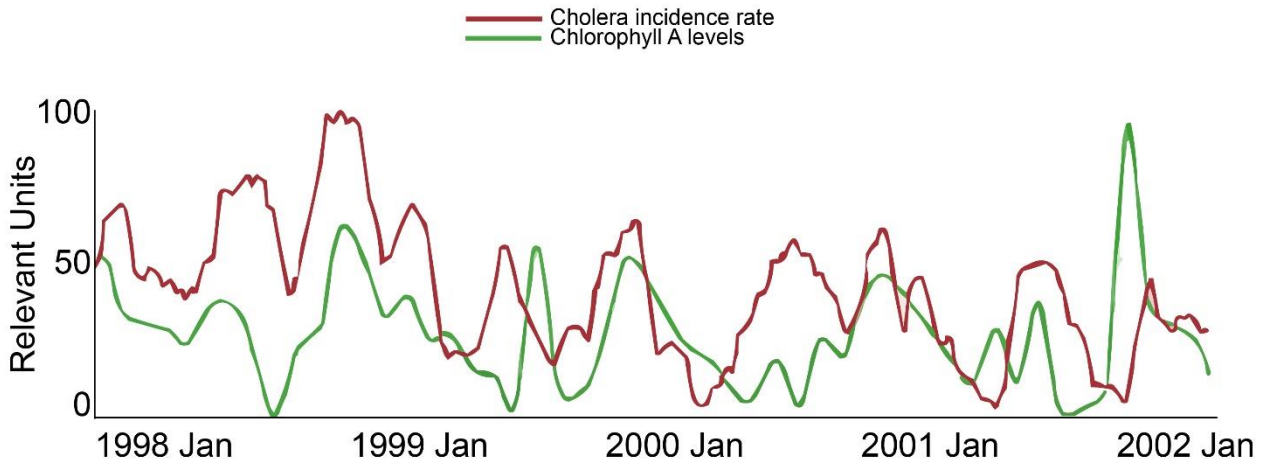

Figure 61. Modeling cholera outbreaks in Bangladesh. Adapted from R.R. Colwell and J. Calkins, unpub. data.

Vibrios constitute a significant portion of the bacteria naturally associated with zooplankton, especially chitinous one (Carli *et al.*, 1993; Montanari *et al.*, 1999; Heidelberg *et al.*, 2002; Lutz *et al.*, 2013; Vezzulli *et al.*, 2022). Numerous studies have reported the association of vibrios with zooplankton, most often by colonizing the body surface of these organisms (Lipp *et al.*, 2003; Pruzzo *et al.*, 2008; Vezzulli *et al.*, 2015). The formation of biofilm by *Vibrio* spp. on the exoskeleton of microcrustaceans and other marine organisms may in fact be a survival strategy (Lipp *et al.*, 2002; Huq *et al.*, 2005) because cells can more efficiently use nutrients absorbed into the biofilm matrix. In this way, vibrios can cope with harsh environmental changes, mainly those related to low nutrient concentrations, such as oligotrophic waters of oceans. They can also withstand the action of toxic compounds and exchange useful compounds with other bacteria or hosts.

It is well known that vibrios are able to break down chitin, one of the richest sources of amino carbohydrates in oceans (Borgeaud *et al.*, 2015). The chitin layer on the surface of copepods can be used as a nutrient. The importance of the *Vibrionaceae* family in preventing the sinking of chitin to the ocean floor has long been recognized (Pruzzo *et al.*, 2008), but this polymer is besides a source of nutrients, and has a key role in the life strategies of several members of this genus. Chitin colonization by *V. chloreae* leads to biofilm production, which in turn induces competence, favoring transformation and horizontal gene transfer (Meibom *et al.*, 2005; Wucher *et al.*, 2019), which are important to acquire virulence and antibiotic-resistant genes. The chitin-induced competence in *V. cholerae* controls the killing of non-immune cells through competence-mediated induction of the Type VI secretion system, facilitating the release of DNA from killed cells, making it accessible for horizontal gene transfer in *V. cholerae* (Borgeaud *et al.*, 2015). Chitin also induces natural competence in other *Vibrio* spp. such as *V. fischeri, V. parahaemolyticus*, and *V. vulnificus* (Blokesch *et al.*, 2014). However, vibrios are not only degrading chitin but are considered agents of organic

matter mineralization, playing an important role in organic matter recycling due to their enzymes, which allow them to use a wide variety of substrates (Thompson, JR and Polz, 2006). They can hydrolase carbohydrates, lipids, and proteins and have the capacity to break down polymers such gelatin, collagen, starch, alginate, lignin, and hydrocarbons, contributing to the recycling of carbon and nitrogen in aquatic environments. Additionally, vibrios can produce polyunsaturated fatty acids, essential compounds for the aquatic food web organisms that they cannot synthesize (Sampaio *et al.*, 2022). Vibrios themselves can be a food source for flagellates and thus contribute to the recycling of organic matter in aquatic environments (Beardsley *et al.*, 2003). During adverse environmental conditions (e.g. antibiotic exposure, nutrient limitation) *Vibrio* cells enter a non-sporulating protective dormant state that enhances their survival and long-term persistence called viable but nonculturable (VBNC) (Xu *et al.*, 1982; Almagro-Moreno and Taylor, 2013; Lutz *et al.*, 2013; M Jayakumar *et al.*, 2020). When external conditions become favorable (e.g. nutrient influx, reduction of antibiotics) dormant cells can recover from the VBNC state, a phenomenon also known as awakening or resuscitation. VBNC cells pose a major public health risk, as these pathogens can be found in this state during interepidemic periods, furthermore, they are a difficult to detect and eradicate source of food and water contamination (Almagro-Moreno and Taylor 2013; Lutz *et al*. 2013).

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D) Voyaging Pathogens: Navigating the Oceanic Pathways of *Vibrio's* Global Epidemiology

Introduction:

The epidemiological dynamics of *Vibrio* pandemics infections have been characterized by the abrupt appearance of outbreaks in remote areas where these diseases had not been previously detected, without knowing the routes of entry of the pathogens in the new area. However, there are recent studies that show the link between the appearance of epidemic outbreaks of *Vibrio* and environmental factors such as oceanic transport of warm waters, which has provided a possible mechanism for the dispersion of *Vibrio* diseases globally. Despite this evidence, there is little information on the possible routes of entry and transport of infectious agents from endemic countries to the entire world. In this sense, the recent advances in genomic sequencing tools are making it possible to infer possible biogeographical patterns of diverse pathogens with relevance in public health like Vibrios.

Clearly, human exposure to these pathogens cannot be completely eliminated, but disease incidence can be reduced if environmental conditions that significantly elevate risk are identified and monitored. The main point of monitoring involves bivalve mollusks, such as oysters and mussels, which can concentrate a large part of these microorganisms, being capable of producing an infection by ingestion. Virulent strains of *Vibrio* are a seafood safety concern, and their detection is important anywhere high levels of this organism are found.

Vibrio epidemiology:

Approximately 10 *Vibrio* spp. are known to cause infections in humans, of which *V. cholerae, V. parahaemolyticus, V. vulnificus* and *V. alginolyticus* are considered most significant (Newton *et al.*, 2012; CDC 2016;2019a; Brumfield *et al.*, 2021). The World Health Organization estimates that there are up to 4 million cases of cholera annually worldwide, leading to more than 100,000 deaths (Islam *et al.*, 2018). Additionally, non-cholera *Vibrio* species are among the leading causes of foodborne infections in humans, primarily through the consumption of raw or undercooked seafood. In response, the CDC has enhanced *Vibrio* surveillance programs in the United States, such as the Cholera and Other *Vibrio* Illness Surveillance (2019a) and the Foodborne Diseases Active Surveillance Network (FoodNet)(Tack *et al.*, 2019)

However, surveillance of *Vibrio* infections on a global scale is limited, particularly in developing countries. Due to under-reporting or failures in reporting, differences in reporting procedures, and the absence of a unified international epidemiological system, the true number of global *Vibrio* infections remains uncertain (Baker-Austin *et al.*, 2018). The CDC estimates that there are approximately 130 undiagnosed cases for each reported case of vibriosis worldwide (Newton *et al.*, 2012).

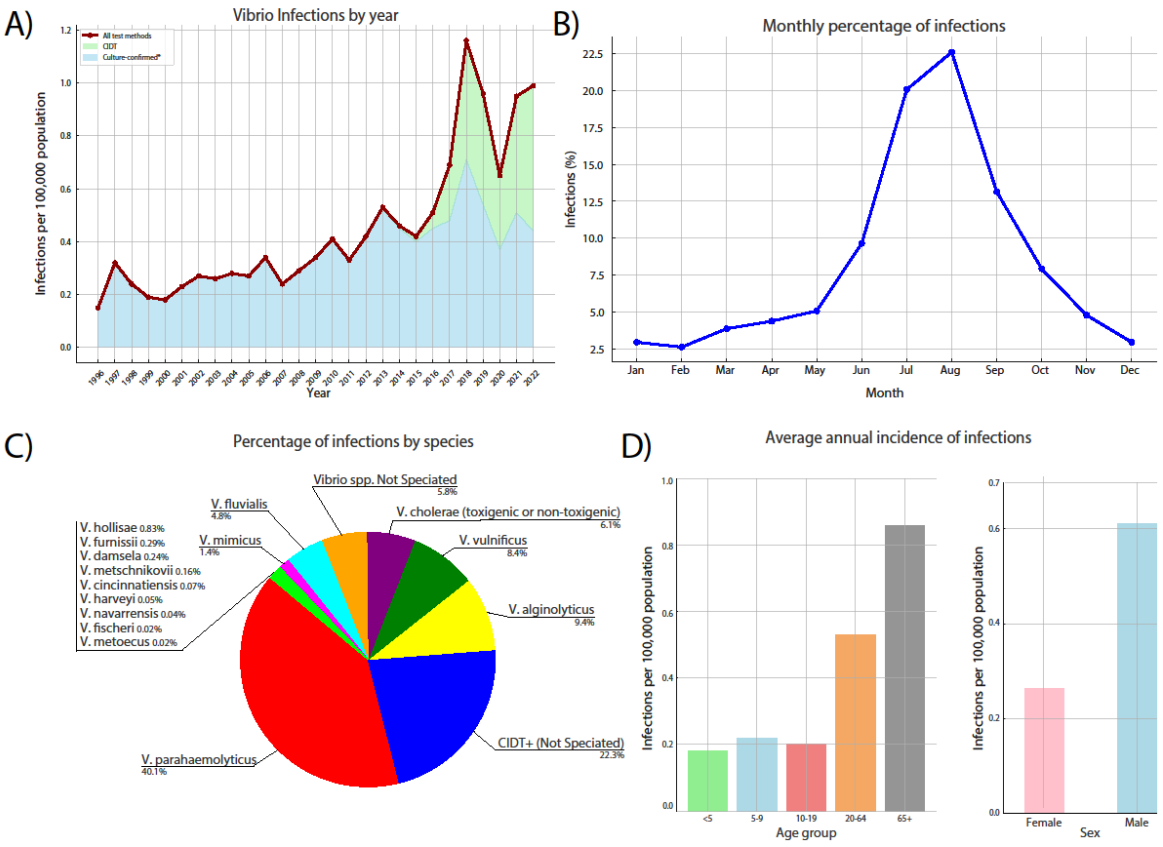

Figure 1 Fig. 1. Pathogen surveillance of infections caused by pathogenic Vibrio spp., 1999–2022 (CDC, 2023). Plots were created using the Foodborne Diseases Active Surveillance Network (FoodNet) Fast to display data for Vibrio infections. A) Vibrio infections by year. Shown is the incidence of infections caused by pathogenic Vibrio spp. B) Infections caused by pathogenic Vibrio spp. presented by month. Shown are monthly percentage of infections across all reported cases. C) Distribution of infections caused by pathogenic Vibrio spp. Shown are percentage of infections caused by pathogenic Vibrio spp. across all reported cases. D) Demographics of infections caused by pathogenic Vibrio spp. The annual average incidence of infections is shown by age (left) and sex (right)

In the United States, Vibrios are estimated to cause 80,000 illnesses, 500 hospitalizations, and 100 deaths each year, of which about 65% are foodborne (Newton *et al.*, 2012). The incidence of foodborne-associated vibriosis nearly tripled between 1996 and 2010 (Newton *et al.*, 2012). According to FoodNet, which monitors 10 states covering 15% of the U.S. population, there has been a long-term increase in reported *Vibrio* infections from 1996 to 2022 (Fig. 1A), with a decrease observed in 2020, likely due to the COVID-19 pandemic restrictions (less consumption of seafood at restaurants and fewer visits to beaches), a trend shown to be common to all major enteric illnesses. Moreover, the percentage of *Vibrio* infections diagnosed using only CIDT (culture-independent diagnostic tests) increased by 56% from 2016–2018 to 2022 (Delahoy *et al.*, 2023).

The majority of *Vibrio* infections occur in warmer months of July and August (Fig. 1B). *V. parahaemolyticus* accounts for nearly half of these infections, while *V. alginolyticus* (approximately 9%), *V. vulnificus* (approximately 8%), and *V. cholerae* (approximately 6%) also play significant roles (Fig. 1C). Notably, most *Vibrio* infections (approximately 60%) occur in males and affect predominately the elderly population over the age of 65 years (Fig. 1D). Of the top 20 most costly marine-borne illnesses 8 are caused by *Vibrio* (Ralston *et al.*, 2011). *V. parahaemolyticus* is responsible for an estimated 34,000 cases annually, costing approximately $40 million. Although *V.*

vulnificus infections are relatively rare (estimated at 100–200 cases annually in the United States), this bacterium is linked to a high mortality rate, exceeding 50% for primary septicemia and approximately 15% for wound infections, with an associated annual economic impact of approximately $320 million (MH Bross, K Soch, R Morales, 2007; Ralston *et al.*, 2011; Scallan *et al.*, 2011; Hoffmann *et al.*, 2015; Muhling *et al.*, 2017). The incidence of vibriosis in the United States tends to increase during warmer years, with an expected rise in frequency and severity of *Vibrio* infections as global warming progresses. A critical concern regarding the risk of *Vibrio* infection is the proximity of some of the largest population centers and economic regions in the United States to coastal areas. Currently, about 40% of the U.S. population resides in coastal counties, a number expected to rise in coming years (NOAA 2021).

Moreover, nearly half of Americans living in coastal counties are considered at elevated risk, including the elderly and low-income households (NOAA, 2021). Seafood remains a vital source of high-quality protein for many populations, with global aquaculture production having increased more than fivefold since 1990 (FAO, 2020). As a result, protein from seafood, particularly aquaculture, is becoming increasingly important for human health and the global economy. Concurrently, an increasing number of environmental *Vibrio* species have been identified as causative agents of vibriosis in aquaculture, leading to significant economic losses (Auguste *et al.*, 2024). A striking example is the early mortality syndrome in shrimp, caused by *V. parahaemolyticus*, which results in annual losses exceeding US$1 billion (Mohammad Jalil Zorriehzahra, 2015). Since *Vibrio* spp. are indigenous to aquatic environments and play significant roles in carbon and nitrogen cycling, it is impossible to completely eliminate the diseases they cause from the environment. Consequently, the implementation of early warning systems is crucial for public health, providing essential information to decision-makers and individuals at high risk of infection (Brumfield *et al.*, 2021; Trinanes and Martinez-urtaza, 2021).

Vibrio pandemics in relation with El Niño:

An aspect of infectious disease, receiving relatively little attention until recently, is the environment (Colwell, 1996). The history of *Vibrio* reveals a remarkably strong association with the sea. The great pandemics followed coastlines of the world oceans (Martinez-Urtaza *et al.*, 2016). *Vibrio* offers an excellent example of how information concerning environmental factors permits better understanding of disease-not only virulence, but equally important, transmission and epidemiology. Recent studies revealed the role of El Niño, a climate pattern that describes the unusual warming of surface waters along the tropical west coast of South America, in disease emergence (Lipp *et al.*, 2002; Martinez-Urtaza *et al.*, 2008; Baker-Austin *et al.*, 2017).

Concerning *Vibrio cholerae*, this evidence with El Niño emerged in 1991 when the seventh pandemic struck South America after a century of absence, first in Peru in the port city of Chancay, 60 km north of Lima. The next day an outbreak was reported from Chimbote, a seaport 400 km north of Chancay. Spread of the outbreak was rapid, and by 7 February 1991, confirmed cases were reported along the Peruvian coast from the Chilean to the Ecuadorean border (Colwell, 1996). In 3 weeks, the epidemic covered > 2000 km of coastal areas and caused 30,000 cases and 114 deaths in the first 7 days. The near simultaneous appearance of cholera along such a great distance of coastline cannot easily or logically be explained by ballast discharge from a single ship in Lima, as was suggested earlier. More likely, the plankton blooms that occurred were triggered by a climatic event, the most logical being El Niño, which brings rain and an influx of nutrients from land and warm sea surface temperatures.

Because phytoplankton blooms can be measured by satellite imagery and zooplankton blooms quickly follow phytoplankton blooms, conditions associated with a cholera outbreak or epidemic could be monitored by satellite (see chapter C) (Ford, 2009). Because a single copepod can carry up to 10^4 cells of V. cholerae, a massive bloom can provide an infectious dose in the brackish water of tidal rivers (Colwell, 1996).

Studies of survival of *V. cholerae* 01 in seawater microcosms revealed that it had the capacity to remain in the culturable state in seawater for a relatively long time, that is, sufficiently long to be carried by ocean currents to widely distant geographical locations (Munro and Colwell, 1996). Other studies showed that, when confronted with high concentrations of carbohydrate, but nitrogen and phosphorous limitation, *V. cholerae* enters the viable but nonculturable state (Shiba *et al.*, 1995). Thus, the viable but nonculturable *V. cholerae* could be transported in nutrient poor seawater and, in association with plankton, over several months and thousands of kilometers, depending on currents and tides and contribute to the occurrence of seasonal epidemics because *V. cholerae* can persist for a long time in the aquatic environment, reintroduction of the organism by infected humans is not necessary (Colwell, 1996).

Gil *et al.*, 2004 demonstrated that cholera cases that occurred in Peru during the summer of 1998 correlated with the peak in sea surface temperatures in relation with to the 1997 to 1998 El Niño event. Interestingly, both El Niño events and cholera outbreaks have increased since the 1970s, a pattern characteristic for both Peruvian coastal areas and the Bay of Bengal. This associations provides evidence for the environmental source of cholera, as well as reasons to explain why cholera epidemics are sporadic and erratic (Colwell, 1996). Another study carried out on the coast of Peru has provided some evidence of the role of water movement in the dynamics of pathogenic clones of *V. parahaemolyticus*, demonstrating that the equatorial waters transported by the El Niño phenomenon were responsible for transporting the pandemic clone of *V. parahaemolyticus* from Asia to America in 1997, a journey of more than 14,000 km (Martinez-Urtaza *et al.*, 2008; Gavilan *et al.*, 2023). The dissemination of this pathogen has also been related in this case to the entry of tropical zooplankton trapped in the waters of El Niño. These observations coincide with the results obtained in previous studies that showed that the survival and growth of *V. parahaemolyticus* in the marine environment is closely linked to its association with zooplankton (Kaneko and Colwell, 1973).

According to these latest investigations, the movements of ocean waters from distant areas may be directly related to the introduction of pathogenic populations of *Vibrio* in areas where they had not previously been detected, being the zooplankton (Erken *et al.*, 2015), and more specifically copepods, the most likely candidates to facilitate transportation. In this way, the arrival of oceanic populations to the coast constitutes a permanent source of Vibrios that makes it difficult to differentiate population groups at the local level.

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E) The Rise of *Vibrio* in a changing world

Introduction:

Planet Earth is currently undergoing significant changes on a global scale due to climate change. These environmental perturbations, combined with factors such as overpopulation and pollution, are contributing to the increased spread of pathogenic Vibrios, raising the risk of infections (Gavilan *et al.*, 2023). In general terms, the warming of our planet is particularly impacting marine ecosystems, as the oceans absorb over 90% of the planet's heat (Zanna *et al.*, 2019). Coastal regions are notably affected, experiencing rising temperatures, more frequent extreme weather events, and increasing sea levels. Some areas, like the Baltic Sea, the Mediterranean Sea, and the Northeastern USA, are warming at rates higher than the global average (Karmalkar and Horton, 2021). However, not all marine species are adversely affected by climate change. Interestingly, Vibrios have been recognized as indicators of climate change, benefiting from these altered conditions (Baker-Austin *et al.*, 2017). These changes are leading to two main effects on *Vibrio* populations: an increase in their seasonal abundance, meaning they are present for longer periods, and an expansion of their distribution range towards the poles (Abanto *et al.*, 2020; Trinanes and Martinez-urtaza, 2021; Gavilan *et al.*, 2023).

Vibrio and climate change

Research has demonstrated a link between climate change and an increase in the frequency and intensity of extreme weather events, including heatwaves and heavy precipitation. These conditions can lead to warmer, less saline coastal waters (Ummenhofer and Meehl, 2017). Analogously, marine heatwaves—oceanic episodes of anomalous surface heating—are expected to become more severe in a warming climate (Smale *et al.*, 2019), potentially causing irreversible impact to marine ecosystems (Holbrook *et al.*, 2019; Doni, Oliveri, *et al.*, 2023). Over the last fifty years, the global decline in marine oxygen levels and a tenfold increase in coastal dead zones have drastically disrupted marine life and costal ecosystems (Breitburg *et al.*, 2018). Changes that occur in the aquatic environment can have a significant impact on abundance and distribution of marine microorganisms, including the impact of ocean warming on the emergence and spread of environmental microbial pathogens (Colwell, 1996; Harvell *et al.*, 2002; Lipp *et al.*, 2002; Ben-Haim *et al.*, 2003; Baker-Austin *et al.*, 2013; Le Roux *et al.*, 2015; Vezzulli *et al.*, 2016; Vezzulli, 2023). *Vibrio* spp., known for their rapid growth and environmental sensitivity, were suggested as indicators of climate change (Vezzulli *et al.*, 2016; Baker-Austin *et al.*, 2017). Documented evidence showed a poleward expansion of *Vibrio*, indicating a significant geographic spread and its implications on human health, including an increase in NOVC-related infections—a clear connection between human disease and climate change (Baker-Austin *et al.*, 2013, 2017; Vezzulli *et al.*,2016, 2020).

Estuarine environments, often warm and slightly salty, create ideal conditions for the growth of *Vibrio* species (Johnson *et al.*, 2012; Vezzulli *et al.*, 2013, 2020) and are warming more rapidly than the open seas due to climate change. Conversely, the open ocean, characterized by its lower temperatures, and nutrient-poor conditions, creates an environment less favourable to the growth of free-living *Vibrio*. Nonetheless, during the summer, coastal areas around the world, such as Chesapeake Bay in the eastern United States, the East China Sea near Shanghai, and the Baltic Sea in northern Europe, experience periods of warmer temperatures and reduced salinity, making them hot-spot for risk of *Vibrio* infections (Semenza *et al.*, 2017; Vezzulli *et al.*, 2020). Beyond marine and

coastal ecosystems, cases of non-O1/non-O139 *Vibrio cholerae* (NOVC) infections have been documented in populations living near inland waters in areas not traditionally affected by cholera (Vezzulli *et al.*, 2020). As sea surface temperatures continue to rise, it is anticipated that both the distribution and prevalence of pathogenic *Vibrio* species will expand, especially in areas where they were previously rare or undetected (Trinanes and Martinez-urtaza, 2021; Semenza, 2022). It should also be noted that while certain *Vibrio*, such as *Vibrio splendidus*, can produce virulence factors even in cooler conditions (Lattos *et al.*, 2021), warmer temperatures may act as a catalyst, selecting for strains with greater virulence potential or increased expression of virulence factors, thereby enhancing their ability to invade hosts more effectively (Vezzulli *et al.*,2020). Environmental data from the pre-industrial era, combined with advanced climate models, have led to the development of new monitoring systems. These systems enable the reconstruction of past conditions, understand the present, and predict future environmental conditions affecting *Vibrio* globally (Trinanes and Martinez-Urtaza, 2021). *Vibrio* have expanded globally, reaching areas previously inhospitable to them. Projections indicate that, by 2100, under the least favorable climate scenario, coastal areas suitable for them could extend by 38,000 km, with an increase in seasonal suitability of approximately one month every 30 years (Trinanes and Martinez-Urtaza, 2021), posing significant economic and public health risks. Earth system model simulations and satellite remote sensing have shown a doubling in marine heatwave events from 1982 to 2016, with projections of further increases linked to global carbon emissions (Frölicher *et al.*, 2018). These heatwaves have been associated with *Vibrio* infections, as seen in Finland and Sweden during the 2014 heatwave (Baker-Austin *et al.*, 2013, 2017). A 60-year survey, employing the continuous plankton recorder in the North Atlantic, correlated rising sea temperatures with increased *Vibrio* prevalence and infections (Vezzulli *et al.*, 2016). Extreme precipitation related to climate change, such as that following Hurricane Katrina, has been linked to an increase of *Vibrio* infections, with flash floods reducing water salinity and promoting pathogenic *Vibrio* growth (Esteves *et al.*, 2015). Stormwater runoff can also promote transmission of antimicrobial resistance and virulence associated genes among bacteria (O'Malley *et al.*, 2023). The evidence is clear that *Vibrio* spp. persist under unfavourable environmental conditions by entering the VBNC state (Xu *et al.*, 1982; Shiba *et al.*, 1995), but the effect with more extensive climate change is not yet known (Brumfield *et al.*, 2021).

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Chapter 2: Aims of the Thesis:

Bacteria of the genus *Vibrio* are found in marine and brackish waters around the world and are the main cause of infections and deaths in humans and animals from the marine environment. These bacteria pose an emergent threat to human health due to warming ocean temperatures associated with climate change. In 2020, there were about half a million cases of non-cholera *Vibrio* infections worldwide, with projections along U.S. coasts of a 50-100 percent increase due to sea surface warming over the next 50 years (Sheahan *et al.*, 2022). Human activities and ongoing climate change are driving the shift in the global distribution patterns of *Vibrio* populations (Vezzulli *et al.*, 2016; Gavilan *et al.*, 2023; Vezzulli, 2023). Certain human actions, such as shipping and ballast water exchange, the worldwide trade of aquaculture products, and increased migratory flows between continents, may have wholly or partly contributed to these changes. These activities have intensified over the last few decades, leading to the transfer of water masses and living organisms across continents. Additionally, human-induced changes such as marine heatwaves, El Niño events, which can lead to changes in plankton distribution patterns, or shifts in ocean currents, may also amplify long-distance migrations (Martinez-Urtaza *et al.*, 2016; Frémont *et al.*, 2022). Climate change is reshaping the biogeography of plankton communities in the oceans across all scales, from viruses to mesozooplankton, with ocean currents speeding up due to warming (Richter *et al.*, 2022). These complex, globally interconnected phenomena likely influence the shift in *Vibrio* populations, considering their planktonic nature and their role in the migratory processes of other marine organisms. Nevertheless, the impact of climate change and the role of the oceans in the spread of these pathogens have remained largely unexplained, due to the scarcity of data. Harmful marine bacteria, such as *Vibrio* species, typically exist at low abundance in open ocean environments but represent a reservoir from which epidemics can arise. Sampling microbes in the aquatic environment often represent a bottleneck for downstream analysis and detection of pathogens. Conventional assays for microbiological water testing generally rely on small volumes of water (in the range of 200–1000 ml), which make it difficult to detect low-abundance microorganisms, including pathogens. In contrast, peristaltic and vacuum filtration systems such as high-volume peristaltic pump are used to concentrate picoplankton on membranes bringing back up to 1.000 L of water for single deployment. These systems can be mounted on ships or deployed in fixed monitoring stations for microbes sampling in the ocean (Yang *et al.*, 2022). Furthermore, in addition to point sampling of seawater, continuous sampling systems may also offer valuable approaches for the monitoring of low-abundant pathogens in the ocean (Vezzulli *et al.*, 2022). For example, the Continuous Plankton Recorder (CPR) is a high-speed plankton sampler designed to be towed from ships of opportunity over long distances that was recently employed to sample pathogenic Vibrios over large spatial scales (thousands of miles) (Vezzulli *et al.*, 2021, 2022; Doni, Oliveri, *et al.*, 2023). Among all analysis methods, the most affirmed is next-generation sequencing, which holds great potential for the identification and genetic characterization of rare microbial pathogens (Pascoal *et al.*, 2021). Deep shotgun metagenomic sequencing provides information of the entire genetic content of a sample and, if correctly designed, can potentially provide insight on the presence of pathogenic strains, also enabling the detection of virulence and resistance determinants outside their genome contexts. As a drawback, this approach can hardly detect rare taxa and genes in a metagenome even at large (> 30 Gb per sample) sequencing output, in particular, in samples with complex community composition. Targeted sequencing, wherein background nontarget DNA is partially excluded from samples before sequencing, solves this problem and reduces the overall costs of sequencing and data analysis per sample. This technique typically involves hybridization-based capture with target-specific biotinylated probes (Vezzulli *et al.*, 2017).

For a complete review about the new approaches based on improved sampling strategies and novel analytical methods offering increased accuracy, high throughput, and informativeness to study and detect microbial pathogens in the marine environment see Doni, Martinez-Urtaza and Vezzulli 2023. By developing and applying new molecular protocols and bioinformatics pipelines for the analysis of marine plankton samples and large metagenomic data collected from global ocean sampling efforts such as the Continuous Plankton Recorder (CPR) survey and the TARA Ocean Expedition this study aimed at providing new insights into the distribution, ecology and connectivity of *Vibrio* populations across the global ocean.

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Chapter 3: Deciphering the Hidden Ecology of *Vibrio* in the Oceans

Abstract:

Long-range dispersals of marine bacteria in the oceans have remained largely indecipherable, which is particularly relevant for *Vibrio*, responsible for global epidemics in humans and animals. Combined analysis of 40 Tbases of metagenomic and satellite-tracked surface drifter data, generated from across the globe revealed that *Vibrio* are abundant members of the ocean surface and presented a strong association with microplankton, which governed their distribution and connectivity at a planetary scale. Long-distance biological corridors have been identified connecting *Vibrio* populations and potentially pathogenic *Vibrio* strains thousands of kilometers away in a fairly short time, estimated of less than 1.5 years to cross an ocean basin. These findings have deep implications for the demography and population dynamics of *Vibrio* species and the epidemiology of associated diseases.

Main Text:

Introduction

Dispersal heavily influences the geographical structure of genetic variation in bacteria. The connectivity between populations favors gene flow and limits the geographical structuring through random mutation and genetic drift, which erodes barriers for allopatric speciation. In oceans, the absence of physical barriers for dispersal enhances interconnectivity between remote areas. However, microbial communities generally follow a distance-decay pattern (1–4), where the genetic similarity declines as distance increases. This relationship can be weaker when dispersal rates are high, for example when currents and vectors (which in turn can be carried by currents) facilitate dispersal. While limited dispersal ability (5) or constrains in response to spatially structured environmental (6, 7) can have the opposite effect. These factors shape the biogeographical patterns and connectivity across oceans. *Vibrio* spp. are natural constituents of estuarine and marine environments and occupy a broad range of ocean ecosystems, from surface to deep waters (8), although their global biogeography and demography have remained unexplored. Among the 150 *Vibrio* species, a dozen can cause infections in humans (9) and many more in animals (10). V. cholerae and V. parahaemolyticus represent the only two known marine bacterial pathogens responsible for global-scale epidemics, which are currently expanding geographically and temporally (11). These pathogenic species are adapted to coastal conditions and their distribution and abundance are driven by physiochemical factors, especially warm and low salinity waters. They are characterized by high genetic intra-species diversity and dynamic population structure, with pathogenic variants emerging and causing infections far away from their endemicity areas through unknown routes and mechanisms of dispersal. Since patterns of transcontinental dispersal has been described for the two pathogenic species (12, 13), the existence of an active oceanic transport of *Vibrio* communities mediated by plankton has been proposed (14–18). The association with plankton could provide protection from the cold saline environments of the open ocean and may represent a food source for survival during prolonged journeys (19). Long dispersal may contribute to recurrent incursions of foreign populations, fostering frequent admixture processes with local populations and introducing constraints on niche specialization (19–21). Despite such evidence, it has been conventionally assumed that *Vibrio* is rarely abundant in the ocean and when present, their scarcity prevents them from being detected in most of the metagenomic studies (22). Consequently, the dispersal via marine transport through the oceans has been considered implausible. Plankton is transported across world oceans by major trans-oceanic currents, which define its global-scale biogeography (23). We hypothesized that the migratory routes defined for plankton could also act as a mechanism for the long dispersal of plankton-attached *Vibrio* across the oceans, enhancing the recurrent introductions of *Vibrio* populations originated in distant areas. These processes would have substantial implications for the demography, population dynamics and evolution of *Vibrio*.

Results:

Bacterial community and Vibrio distribution in the oceans

The taxonomic analysis of bacterial communities utilizing Kraken2 (24) with the NCBI bacteria refseq database (Fig. 1A), revealed distinct structures in the oceans. Candidatus Pelagibacter showed the highest average abundance (6.4%), ranging from 18.3% in the Arctic Ocean (ARC) sample to 3.2% in the Mediterranean Sea (MED). Prochlorococcus, Synechococcus and Alteromonas showed mean abundance around 5%, with higher abundances in the temperate regions and lower in polar regions. *Vibrio* was the seventh most abundant bacterial genera, with the highest abundance (2.6%) observed in the North Indian Ocean (ION) and the lowest (1.2%) in the Southern Ocean (SOC), with an overall average value of 1.8%. Interesting, *Vibrio* was significantly more abundant in the plankton-attached fraction compared to the free-living fraction (Fig. 1B) (Wilcoxon $p < 0.001$) and was more prevalent in superficial layers than in deeper waters (Kruscal-Wallis $p < 0.001$) (Fig. 1C).

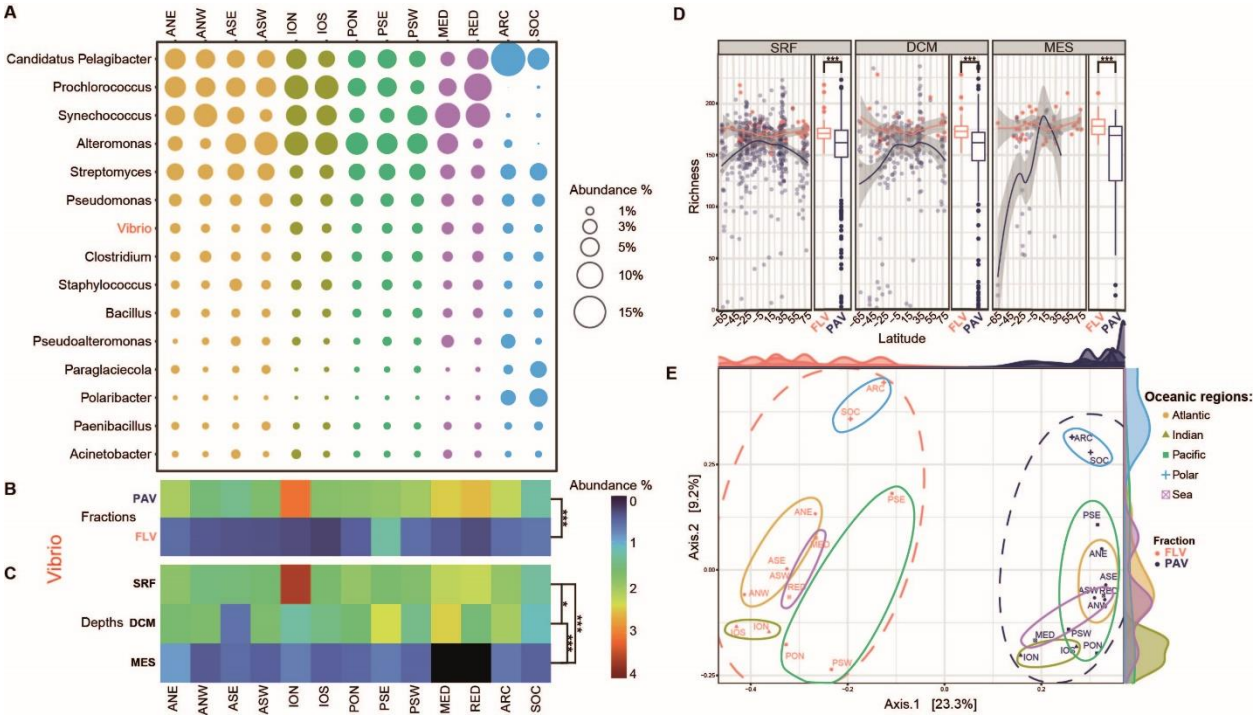

Fig. 1. Bacterial composition of oceanic samples. (A) Bubble plot showing the top 15 bacterial genera divided for oceanic regions (columns) with Vibrio in the 7th position. Oceanic regions include: ANE (Northeast Atlantic Ocean), ANW (Northwest Atlantic Ocean), ASE (Southeast Atlantic Ocean), ASW (Southwest Atlantic Ocean), ION (North Indian Ocean), IOS (South Indian Ocean), PON (North Pacific Ocean), PSE (Southeast Pacific Ocean), PSW (Southwest Pacific Ocean), MED (Mediterranean Sea), RED (Red Sea), ARC (Arctic Ocean) and SOC (Southern Ocean). Heatmap for Vibrio: (B) the average of the frequencies of Vibrio in the PAV (plankton attached fractions) which correspond to 5-20 µm, 20-180 µm, 180-2000 µm and the FLV (free-living fraction) which correspond to 0.22-3 µm; (C) shows the average frequencies of Vibrio across the depths: SRF (Surface waters), DCM (Deep Chlorophyll Maximum) and MES (Mesopelagic). Black squares mean no data (D) Vibrio alfa diversity calculated as the richness of Vibrio species across samples and plotted against the latitude of the stations for the different depths: SRF, DCM and MES. (E) 31-mers based PCoA (k-PCoA) of the Vibrio across the samples, (colors indicate fractions and oceanic regions); the percentage of the variation explained by each axis is indicated in parentheses after the axis label.

From a subsequent Kraken2 reclassification using the "Enterobase *Vibrio* database" (25), about 160 million *Vibrio* reads were obtained. Alpha diversity, calculated as richness, showed different latitudinal (Fig. 1D) and longitudinal (fig. S1) patterns. Indeed, the overall richness for free-living *Vibrio* (FLV, 0.22-3 µm) was significantly higher than that of the plankton-attached *Vibrio* (PAV) (Wilcoxon $p < 0.001$). Beta diversity analysis, based on the 31-mers frequencies (k-PCoA) (Fig. 1E), clearly separated samples between FLV and PAV in the first axis (23.3% variation). Meanwhile, in the

second axis (9.2% variation) for the FLV, the oceanic regions were well defined, while in the PAV there was an overlap between them, suggesting a less structured biogeography caused by a potential connectivity between the oceanic regions. Permutational multivariate analysis of variance (PERMANOVA) confirmed that both size fraction ($p < 0.01$) and in a lesser extent the oceanic regions ($p < 0.05$), influenced significantly the *Vibrio* communities. The beta diversity analysis with the *Vibrio* kraken2 taxonomy abundances (t-PCoA) (fig. S2), exhibited similar patterns to the k-PCoA ordination. Indeed, the mantel test between the two distance matrices revealed a strong and significant correlation ($r = 0.8$, $p < 0.01$). The strong separation between PAV and FLV could have important biological implications for the structure of *Vibrio* populations. For this reason, the biogeographical structure for each size fraction was subsequently evaluated.

Vibrio biogeographical structure

Vibrio biogeographical structures were identified based on the similarity values from their kmers sequence content for each fraction from superficial water samples. PAV were further separated in attached to: nanoplankton (NAV 5-20 µm), microplankton (MiAV 20-180 µm) and mesoplankton (MeAV 180-2000 µm) (26).

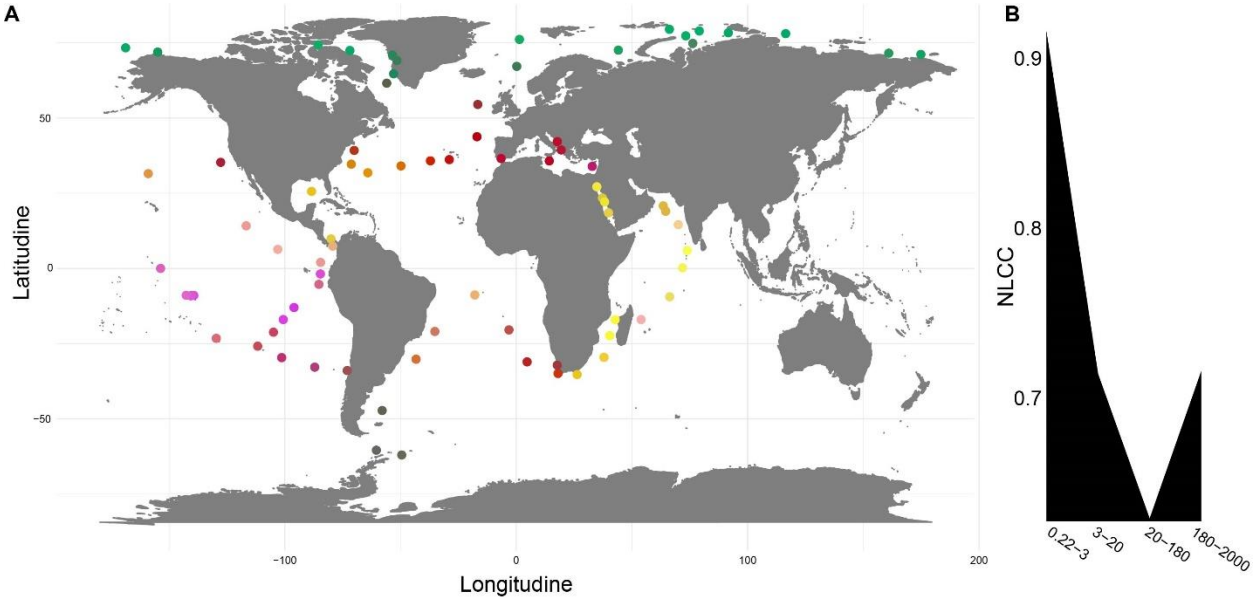

Fig.2 Vibrio biogeographical structures of surface stations, for the FLV (0.22–3 µm) fraction (A), colors of the stations are derived from the RGB-PCoA based on Vibrio metagenomic dissimilarities, similar colors indicate similar communities. (B) The nonlinear correlation coefficient (NLCC) calculated for each size fraction based on the differential patterns in the RGB-PCoA stations.

The obtained Bray-Curtis 31-mers matrices of each fraction were used to produce RGB-PCoAs, where the stations color was based on their position in the ordination. Subsequently the biogeographical structures were visualized plotting the stations with their coordinates, maintaining the colors of the RGB-PCoAs (fig. S3). The FLV fraction (Fig. 2A) had the most clearly defined biogeographical patterns, in line with the k-PCoA result. The nonlinear correlation coefficient (NLCC) was used to quantify the biogeographical structures detecting if stations had a spatial nonlinear pattern in each RGB-PCoA. Interesting, as the size of filtration increased, the biogeographic compartmentalization decreased, except for MiAV, which had the lowest NLCC (Fig. 2B). These results suggested that plankton had a relevant role in the formation of *Vibrio* communities spatial patterns, which may be related to their role in facilitating *Vibrio* large-scale displacement.

Vibrio and oceanic circulation

To investigate if *Vibrio* displacement were related to plankton transportation across the ocean currents, the estimated travel time (TT) (table S1) and Langrangian trajectories among stations were computed using the NOAA Global Drifter Program, that comprises observations of ocean surface currents from a network of drifting buoys. TT has previously been proposed as a robust approach for studying dispersal mechanisms in oceans (23, 27). The similarities (table S2-S5) derived from the 31-mers Bray-Curtis dissimilarity matrices of samples collected exclusively from surface waters for each size fractions (FLV, NAV, MiAV and MeAV), were compared with TT calculating the cumulative Spearman correlations (Fig. 3A).

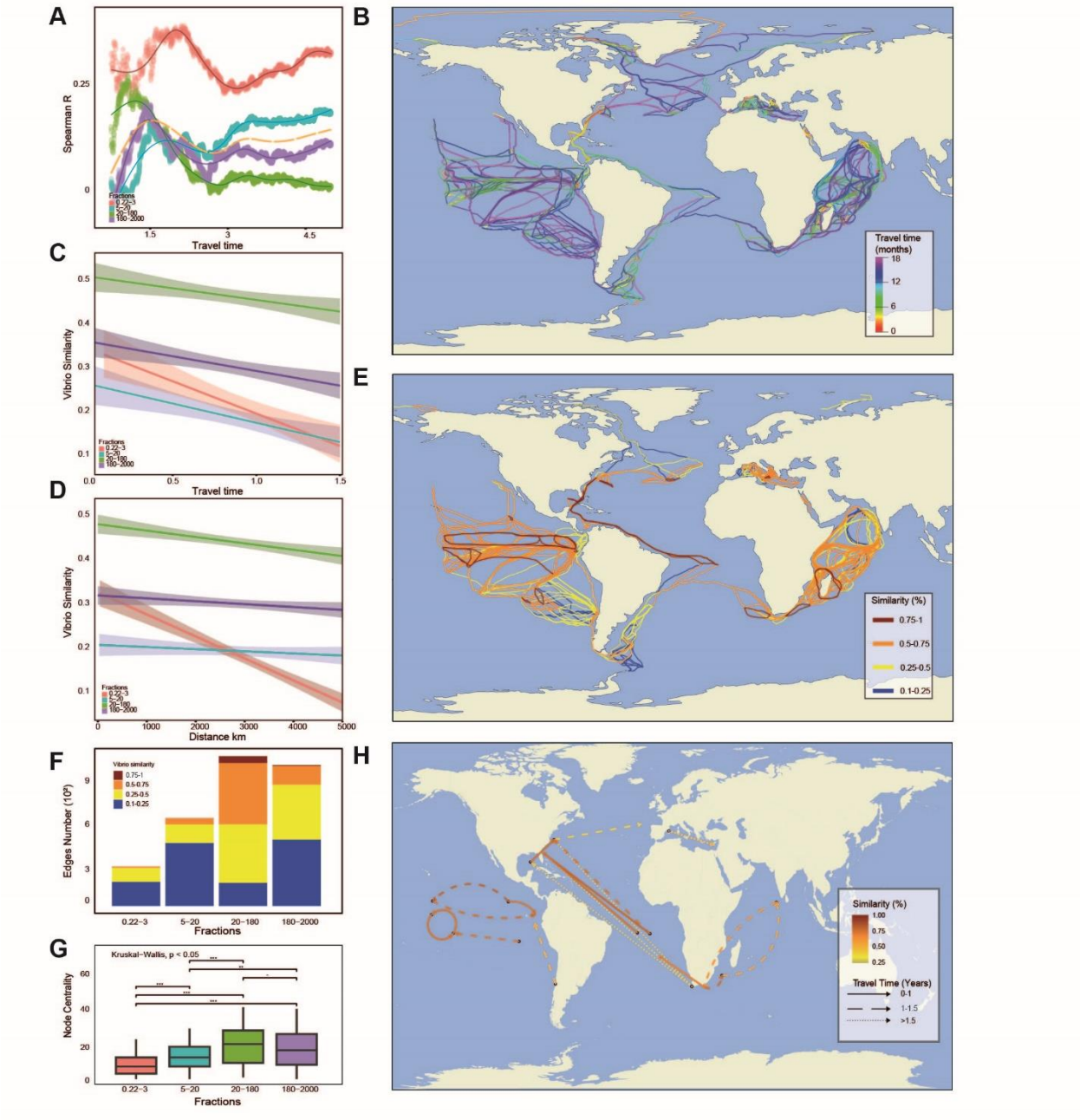

Fig3 Vibrio surface dispersion through Oceans. (A) Spearman cumulative correlations between Vibrio diversity and travel time (TT) for each different size fraction. Yellow-dashed line represents the overall trend. (B) Lagrangian trajectories corresponding to the minimum travel time, referred as colors of the edges, among all stations connected by a maximum of 1.5 years. (C) Spearman correlations between Vibrio similarity and travel time. (D) Spearman correlations between Vibrio similarity and geographic distance in km without crossing lands. (E) Network among stations connected by a travel time less than 1.5 years for the MiAV fraction (20-180 µm), connections are color-coded based on the similarity of the Vibrio populations between stations. (F) Frequency of edges and

their strength of similarity for each fraction (FLV: 0.22–3 µm, NAV: 5–20 µm, MiAV 20–180 µm, and MeAV 180–2000 µm) in their corresponding network. (G) Node centrality values for each fraction in their corresponding network. (H) Long-Distance Oceanic corridors and travel time of Vibrio associated to microplankton (MiAV; 20-180 µm).

Notably, the maximum cumulative correlation values were observed for pairs of stations separated by a TT up to 1.5 years across all size fractions (Fig. 3A yellow-dashed line). This result was consistent with a previous study investigating plankton TT from TARA samples (23). Consequently, this threshold was selected for subsequent analyses (Fig. 3B). For each size fraction, *Vibrio* sequence-kmers similarity was correlated with TT up to 1.5 years (Fig. 3C). Fisher z-test was employed to determine whether these correlations differed among fractions. This analysis revealed significant ($p < 0.05$) differences between the FLV and the other fractions (table S6), suggesting implication of plankton for the dispersion of *Vibrio* following major oceanic currents. Interestingly, the highest correlation values were observed for the MiAV and the slopes exhibited an inverse relationship with the size fraction, decreasing as the fraction size increased except for the MiAV (Fig. 3C). The same approach was employed to correlate pairwise similarity with distance up to 5000 kilometers (Fig. 3D), a scale distance in the order of the distance to cross an oceanic region (23). The Fisher z-test revealed significant ($p < 0.05$) differences in correlation for all combinations except for comparison between the MiAV and MeAV fractions (table S7). When comparing the correlations between similarity and TT as well as similarity and distance in kilometers (fig. S4), the correlation slopes of FLV and NAV fractions showed a similar decreasing trend in both analyses. In contrast, the correlation slopes between similarity and distance in km for the MiAV and MeAV fractions were close to 0, indicating a weak correlation. However, their correlations of similarity and TT exhibited negative slopes and a higher similarity compared to FLV and NAV (fig. S4). These results support that PAV dispersions across oceanic regions are primarily influenced by water currents and less by geographical distance. Whereas, the FLV showed a comparable negative trend in both the kilometer and TT correlations, which can be ascribed to the fact that the dispersion of the FLV does not rely on plankton for transportation. The analysis of the *Vibrio* similarity network, focusing on pairs of stations connected by TT of less than 1.5 years, revealed clear differences among size fractions. MiAV had the highest number and strength values of edges (corresponding to the sequence similarity) in the network (Fig. 3E), while FLV network showed a reduced number and strength of edges, which were weaker compared to also the other fractions (fig. S5, Movie S1). The barplot (Fig. 3F) summarized the number of the edges connecting stations and their strength, across the different size fractions. Comparing FLV and MiAV, the weak similarity range (<0.25) decreased by 3.87%, while the medium-weak (0.25<0.5) and medium-strong similarities (0.5<0.75) increased respectively by 316.04% and 5675.00%. Notably, the highest range of sequence similarity (0.75-1) was present only in the higher plankton-associated fractions (MiAV and MeAV). A partial mantel test, correlating the Bray-Curties kmers matrix of each fraction with the Euclidean matrix based on the surface water temperature and salinity, as proxies for local conditions, indicated that such conditions exerted a stronger influence on the FLV ($R=0.8$, $p < 0.05$) compared to the other fractions: NAV ($R = 0.2$, $p < 0.05$), MiAV ($R = 0.5$, $p < 0.05$), and MeAV ($R = 0.1$, $p < 0.05$). FLV showed adaptation to local environmental conditions and biogeographical structure, probably defined by isolation for weak dispersal, which may provide a certain level of ecological cohesion. Notably, node centrality values, representing the connectivity of each station within the network, for PAV were significantly higher (Fig. 3G). Regions of high centrality provided information about stations with a higher degree of reachability (28), which may promote gene flow and reduce genetic differentiation (i.e., hubs for

genetic connectivity, (29)). Most of these stations were in the Indian and Pacific Oceans (fig. S6 A and B) and they played a crucial role in facilitating large scale dispersal of *Vibrio* populations throughout the marine environment. The MiAV high connectivity hubs had a close correspondence with previously described transcontinental transmission routes for pathogenic *Vibrio* (12). Moreover, the MiAV long-distance biological corridors (Fig. 3H, table S8) were identified as connections between stations linked over long distances, with high similarity and fast TT, spanning one or more oceanic regions. These corridors showed an interesting patterns of *Vibrio* circulation in the major hubs of connectivity such as Indian ocean and Pacific Ocean. For instance, between ION and South Indian Ocean (IOS) the estimated TT was of 1.1 years with a similarity of 0.7. In the Pacific Ocean, connection from south to the equator had a TT of 1.3 years and a similarity of 0.5. Circulations within Pacific Ocean were identified, one connected the Southeast Pacific (PSE) and Southwest Pacific (PSW) Oceans with a similarity of 0.8 and a TT ranging from 0.9 to 1.2 years, while the second connected the North Pacific Ocean (PON) to PSE with a similarity of 0.8 and a TT ranging from 0.6 to 1.4 years. Detailed analysis of the MiAV average TT and similarities for intra- and inter-oceanic regions are shown in fig. S7. However, relevant results showed a high average similarity between Northwest Atlantic (ANW) and Northeast Atlantic (ANE) oceans with an average of 0.7 in both directions and an average time travel of 1.31 years from West to Est and 1.25 years in the opposite direction. From Southeast Atlantic (ASE) to Southwest Atlantic (ASW), the average similarity was 0.71 while the TT was 0.95 years. Among intra-oceanic regions, ASE had the highest similarity of 0.65 with an average TT of 0.69 years.

Vibrio species

Vibrio reads from both FLV and PAV, for each oceanic region, were co-assembled to achieve a higher taxonomic resolution. Co-assembly was performed by grouping stations into oceanic regions based on Longhurst Provinces and station proximity (30). A total of 367808 contigs were produced, with an average of 14417 contigs per oceanic region and overall average N50 of 684 bp (table S8). The contig taxonomy analysis identified approximately 200 *Vibrio* species (table S9), with an overall mean confidence of 0.95 and for the potential pathogens V. cholerae and V. parahaemolyticus of 0.97 and 0.96.

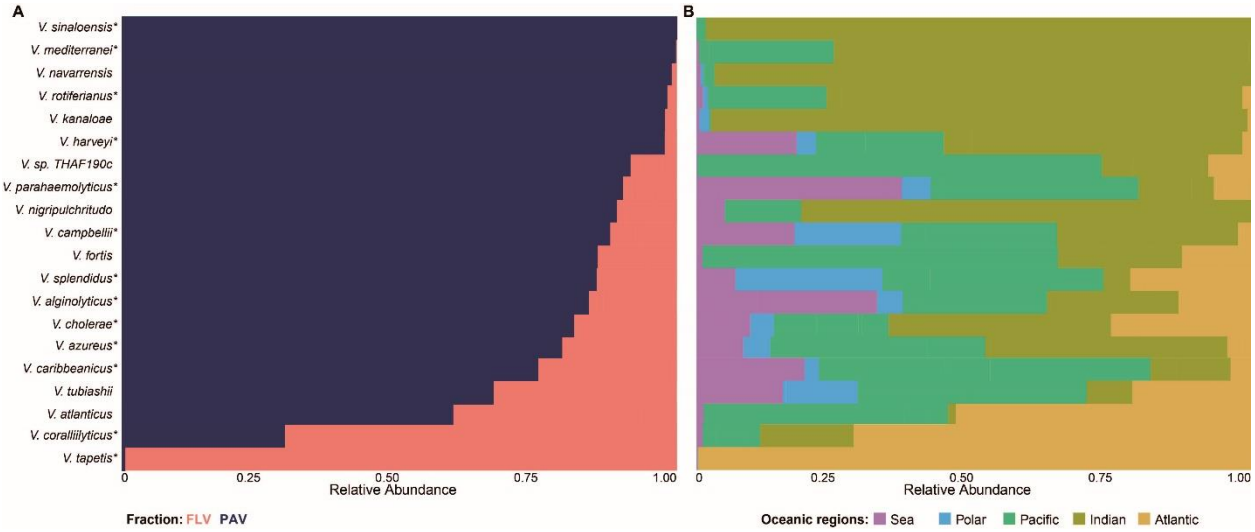

*Fig 4 Top 20 Vibrio species distribution, (A)Distribution of Vibrio species between the free-living (FLV) and plankton attached (PAV) fractions. Species with specifically different abundance related to fraction indicated with *. (B) Distribution of Vibrio species among oceanic regions.*

Interestingly, *Vibrio* species known as causal agents of both human and animal diseases, generally exhibited higher abundance in the PAV (Fig. 4A). Notably, among the 13 species significantly associated with plankton, some were potentially pathogens for humans (V. cholerae, V. parahaemolyticus and V. alginolyticus) and for animals (V. harveyi, V. rotiferianus, V. campbellii, V. mediterranei, V. sinaloensis and V. splendidus). While different patterns were shown for species distribution in oceanic regions (Fig. 4B). This result confirmed the role of plankton in the dispersion of *Vibrio* species, highlighting its role for the transport of species which could have implications for human and aquatic animal health.

Discussion

The present study has refuted the conventional assumption that the open ocean is a hostile environment to most *Vibrio* species, revealing them as natural inhabitants of the pelagic ecosystems. *Vibrio* communities prevailed in the upper ocean layers, existing as free-living organisms but, predominantly associated with plankton. Distinct niche preferences were revealed by both taxonomic distribution and sequence similarity, highlighting the importance of plankton to structure the *Vibrio* communities in ocean. FLV showed niche adaptation and biogeographical structure, probably defined by weak dispersal and isolation, which may provide ecological cohesion. These results are consistent with previous studies, suggesting that microbes evolve faster in the absence of effective dispersals than the effect of the dilution caused by ocean currents, leading to distinctive biogeographic patterns (31). Microorganisms with a lower dispersion have higher richness (32), as observed for FLV compared with the PAV. The identification of a strong distance-decay in FLV supports this evolutionary context, where neutral evolution and genetic drift may sustain the observed geographical distribution. On the other hand, environmental factors had a substantial importance to shape the genetic diversity and similarity in populations with low dispersal ability (FLV) compared to those undergoing long-range dispersions (PAV), which is consistent with previous studies supporting the role of the dispersal ability in modulating the relevance of environmental factors (6, 7). In contrast, PAV, representing the majority of *Vibrio* in the oceans, showed different population structure and dynamics, with a more diffuse biogeographical distribution and a weaker distance-decay pattern, which supports the existence of an active dispersal across oceanic regions. Unlike FLV, dispersal of PAV was more influenced by oceanic transport than by geographic distance. Thus, PAV and FLV populations may evolve under different constraints (33). While there were evident differences between FLV and both MiAV and MeAV patterns, the NAV fraction was like a transitional group between these two major distinctions, as already suggested for *Vibrionaceae* family (34). The connectivity observed for *Vibrio* populations was consistent with the plankton transoceanic migration routes connecting distant regions (23). Results of the present study provide robust evidence of a plankton size-dependent effect on the interconnectivity of *Vibrio* populations globally, following the dominant trajectories of the oceanic currents and plankton migrations. Because TT-based trajectories correspond to the dominant oceanic pathways, which transport the largest volume of ocean water and its contents, such as plankton, nutrients, and heat. Indeed, TT is not only considered a proxy for the time required for movement in the ocean, but also for the biotic interactions of what is transported within the currents (23). The effective oceanic transport mechanisms have also been supported by the absence of an isolation-by-distance pattern and weak biogeographical and population structure, consequences of the lack of genetic barriers to exchange between populations. The South Pacific and Indian oceans act as major hubs for the *Vibrio* distribution. Notably, these regions are considered the major hotspots for *Vibrio* diseases in the world (12, 13), with frequent transcontinental epidemic radiations following parallel dispersal trajectories to those described in this study. These observations are also consistent with results obtained from the analysis of the global collection of V. parahaemolyticus, which concluded that the high diversity and low differentiation of these populations resulted from the long-range dispersals and local admixing of populations (35). The ocean circulation delineated in this study ensures the migration and dispersal of *Vibrio* at a planetary scale following the major long-distance biological corridors in the oceans. This continuous admixture, through the introduction of *Vibrio* strains from distant locations with local populations in a fairly short time, might be responsible for the

establishment of environmental reservoirs and the emergence of new pathogenic variants and disease outbreaks under favorable conditions. This is corroborated by the frequent colonization events, documented for *Vibrio* globally (13, 35), which counteracts the biogeographic effects on population structure, imposed by drift and/or selection, eroding the distance-decay relationship. This work revealed the importance of plankton acting as genetic draft for *Vibrio* and as driver for the dissemination of potential human pathogens, such as V. parahaemolyticus, V. cholerae and V. alginolyticus, across oceans. *Vibrio* is considered one of the most responsive organisms to climate change induced conditions in the ocean (11, 36). The increasing warming trends are providing suitable ecological conditions for the colonization of a growing number of regions worldwide (37). This favours the introduced populations for a successful establishment and the subsequent incorporation in the local communities (19, 35, 38), as shown by the poleward expansion of *Vibrio* infections in the Northern Hemisphere (37, 39). However, climate change is affecting the oceans in multiple ways, including speeding up and modifying the routes of the major ocean currents (40) and altering the biogeographical patterns of plankton distributions (41). As shown in this study, both aspects are intrinsically linked to the dispersal dynamics and connectivity of *Vibrio* populations and govern the microbial community assembly across spatial scales. In consequence, we foresee substantial changes in the global demography of *Vibrio* populations in the future resulting from the conditions imposed by climate change, with collateral effects on the epidemiology of *Vibrio*-related diseases.

Supplementary

Materials and methods

TARA metagenomes

The general aim of the TARA Ocean expedition was to assess the complexity of ocean life across comprehensive taxonomic and spatial scales, sampling the oceans world-wide following standardized protocols for the collection and for data production (26). The methodology used for the sampling, size fractionation, DNA extraction and shotgun metagenomic sequencing has been already extensively described (26, 42, 43). However, for each station, usually water from three different depths was sampled: surface, deep chlorophyll maximum and mesopelagic waters. Then, the samples were serially filtered, with a prokaryote-enriched fraction (FLV: 0.22–3 µm) and four eukaryote-enriched fractions (NAV: 5–20 µm, MiAV: 20–180 µm and MeAV 180–2000 µm). In this study, about 1500 shotgun metagenomes of 210 TARA stations belonging to 4 NCBI TARA Oceans project (PRJEB1787, PRJEB4352, PRJEB9740 and PRJEB9691) were downloaded and analyzed. Data corresponded to approximately 40000 Gbases of fastq files for a total of 3 billion reads. All the scripts used for the analysis of the downloaded metagenomes can be found at: https://github.com/Luponsky/Deciphering-the-Hidden-Ecology-of-Vibrio-in-the-Oceans and a brief workflow of the pipeline is shown in fig. S8.

Metagenomic reads analysis

Metagenomic reads were quality checked with trimgalore v0.6.7 (44). The analysis of the bacterial composition was conducted with Kraken2 v2.1.2 (24) using the Refseq NCBI Bacteria database (downloaded in 2021). The frequencies of bacterial genera were normalized using Bracken v2.6.2 (45). The top bacterial genera in oceans were visualized as a bubble plot. *Vibrio* genus average abundances across different fractions and depths were highlighted in the heatmaps. Both plots were generated using the R package GGplot2 (46). Metagenomic reads classified as *Vibrio* were extracted using the extract_kraken_reads.py tool from KrakenTools (47). These reads were subsequently reclassified using a custom Kraken2 database, generated with the *Vibrio* genomes present in "The *Vibrio* database" from Enterobase in 2021 (available at: https://enterobase.warwick.ac.uk/species/index/vibrio, (25)), following the Kraken 2 manual. The frequencies of *Vibrio* species were normalized using Bracken. The subsequent analyses were performed in R using Phyloseq package (48) for analysis and GGplot2 for the visualizations (46). The *Vibrio* alpha diversity, estimated as richness, was estimated using *Vibrio* species frequencies transformed into presence absence and then plotted against latitude and longitude for each specific depth. Beta diversity was visualized as a PCoA ordination based on the Bray-Curtis dissimilarity matrix calculated from the *Vibrio* species abundances (t-PCoA). Beta diversity based on Bray-Curties dissimilarity matrix was also assessed using kmers (k-PCoA) calculated with Simka (49). *Vibrio* kmer frequencies were calculated using 31-mers and filtering low complexity reads. PERMANOVA with 9999 permutations from adonis2 of Vegan package (50) was used to compute the model for the interaction of the factors (~ Fractions* Oceanic Regions). Metagenomic dissimilarity based on 31-mers, has been shown to be genome/species specific (51) and a reliable kmer size to evaluate differences in the genomic identity of organisms (23). To assess the correlation between the two

Bray-Curtis dissimilarity matrices (based on taxonomy counts and kmers), a mantel test was performed in R using mantel function of Vegan package (50).

Vibrio biogeographical structure

Vibrio biogeographical structures were evaluated for each size fraction (i.e., 0.22-3 µm, 5-20 µm, 20-180 µm, and 180-2000 µm) based on the PCoA ordination of the Bray-Curtis 31-mers dissimilarity calculated with Simka, from stations collected only in the surface waters and filtering low complexity reads. Thus, an RGB-PCoA was produced by assign to each station a color based on its position in the ordination, taking account of the axis variation and the spatial position of the first three axes to map the stations into RGB color values. Thus, stations with similar *Vibrio* communities shared the same range of colors. The RGB-PCoA were used to calculate the heuristic nonlinear correlation coefficient (NLCC) using the 'nlcor' R package (available at: https://github.com/ProcessMiner/nlcor), which can detect no linear pattern on the distribution of the stations composing the RGB-PCoA. The heuristic method involves adaptively identifying multiple local regions of linear correlations to estimate the overall nonlinear correlation. Finally, the stations were plotted on a map based on their geographical coordinates, with each station colored using the relative RGB color.

Ocean circulation and correlations with *Vibrio* diversity

In order to assess the oceanic connectivity among stations, the DrifMLP package (52) was used. The Six-hourly Interpolate Database (at: https://www.aoml.noaa.gov/phod/gdp/interpolated/data/all.php) of the Global Drifter Program, that comprises observations of ocean surface currents from a network of drifting buoys was used as input to estimate the connectivity between stations. By tracking the Lagrangian trajectories of drifters among stations, the potential pathways were obtained, revealing their connectivity patterns and travel time. Following the package recommendations, the bootstraping and grid rotation to estimate the uncertainty in the trajectory and travel time estimates were applied. Each *Vibrio* Bray-Curtis 31-mears dissimilarity matrix, divided by size fractions (i.e., 0.22-3 µm, 5-20 µm, 20-180 µm and 180-2000 µm) selected from samples collected in the surface waters, was used to compute the cumulative correlations between *Vibrio* kmers diversity and travel time using Spearman rank correlation coefficient. Based on the result of this analysis and previous results from analogous analyses using TARA samples (23) and from an ecological perspective, we determined a travel time cutoff of 1.5 years between stations. The travel time of 1.5 years corresponds time needed to travel across an oceanic basin or gyre (53). Subsequently, Spearman correlation using the cor function of stats R package (54) between *Vibrio* kmers similarity and travel time up to 1.5 years and the Spearman correlation between *Vibrio* kmers similarity and the distance within 5000 kilometers (which is on the order of the distance to cross an oceanic gyre (23)) were calculated. Distance in km among stations was calculated using searoute.py (available at: https://github.com/genthalili/searoute-py), a python package for generating the shortest sea route between two points avoiding land using the Haversine formula. Fisher z-test from diffcor R package (55) was applied to find if there were significant differences among the correlations of the four fractions of both Spearman correlations analysis. Moreover, to compare both the correlations of

Vibrio similarity among travel time and distance in km, the comparison of the Z-scores of logarithmically transformed distance and travel time was obtained. The network approach was used to investigate the *Vibrio* kmers metagenomic similarity in relation to travel time, for each fraction, removing outliers. The plot was generated on a global map using Python Cartopy (available at: https://pypi.org/project/Cartopy/) displaying the trajectories of the *Vibrio* similarities connected by station separated up to a 1.5 years of travel time. The longitudes were adjusted to ensure a smooth transition across the antemeridian. The 4-panel video displaying the trajectories for each fraction was produced following similar preprocessing steps used for the static networks. The video was created by updating a set of frames by the trajectories and adjusting the time indicator as the animation advanced and then was saved as an MP4 file with a frame rate of 20 frames per second. *Vibrio* similarities for each fraction were divided in ranges: weak (0.1-0.25), weak-medium (0.25-0.5), medium-strong (0.5-0.75) and strong (0.75-1) and summarized for each fraction as a barplot. Subsequently for estimate the influence of local conditions on *Vibrio* communities, a partial mantel test using the Vegan package was applied to correlate with Sperman the kmers Bray-Curties distance matrix for each fraction to the Euclidean matrix based on the surface water temperature and salinity, as proxies of local conditions, removing the effect of spatial autocorrelation with the Euclidean distance matrix of geographical coordinates of the sampling stations. Temperature and salinity were recovered from the environmental context of all samples from the Tara Oceans Expedition metadata (available at: https://doi.pangaea.de/10.1594/PANGAEA.875579). Network theory metrics were applied to calculate measures of node centrality using igraph R package (56). Statistics of the distribution of node centrality among fractions was computed using Kruskal-Wallis and Wilcoxon tests and plotted with Ggpubr (available at: https://github.com/kassambara/ggpubr). Stations with high node centrality serve as stepping-stones to connect *Vibrio* populations, because they have a higher degree of reachability (28), which in turn may promote gene flow and reduce genetic differentiation levels making them hubs for genetic connectivity (29).

Vibrio species analysis

Metagenomic *Vibrio* reads were co-assembled for each oceanic region, using geographically related stations, following the approach previously used for other TARA Oceans metagenomic co-assemblies (30). This was done for both FLV and PAV using megahit (57). Taxonomy classification was assigned with Contig Annotation Tool (CAT) (58) using the NCBI nr database. The relative abundance of *Vibrio* species was calculated as Contigs Per Million, mapping *Vibrio* reads on the *Vibrio* contigs using Salmon (59) with the metagenomic (—meta) flag, for FLV and PAV for each oceanic regions and was normalized with relative log expression method (60) using MicrobiomeMarker R package (available at: https://github.com/yiluheihei/microbiomeMarker). The distribution of the top 20 *Vibrio* species was visualized between fractions and among oceanic regions. Pairwise Mann-Whitney test was used to find if there were significant differences of species distribution between FLV and PAV.

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Supplementary figures:

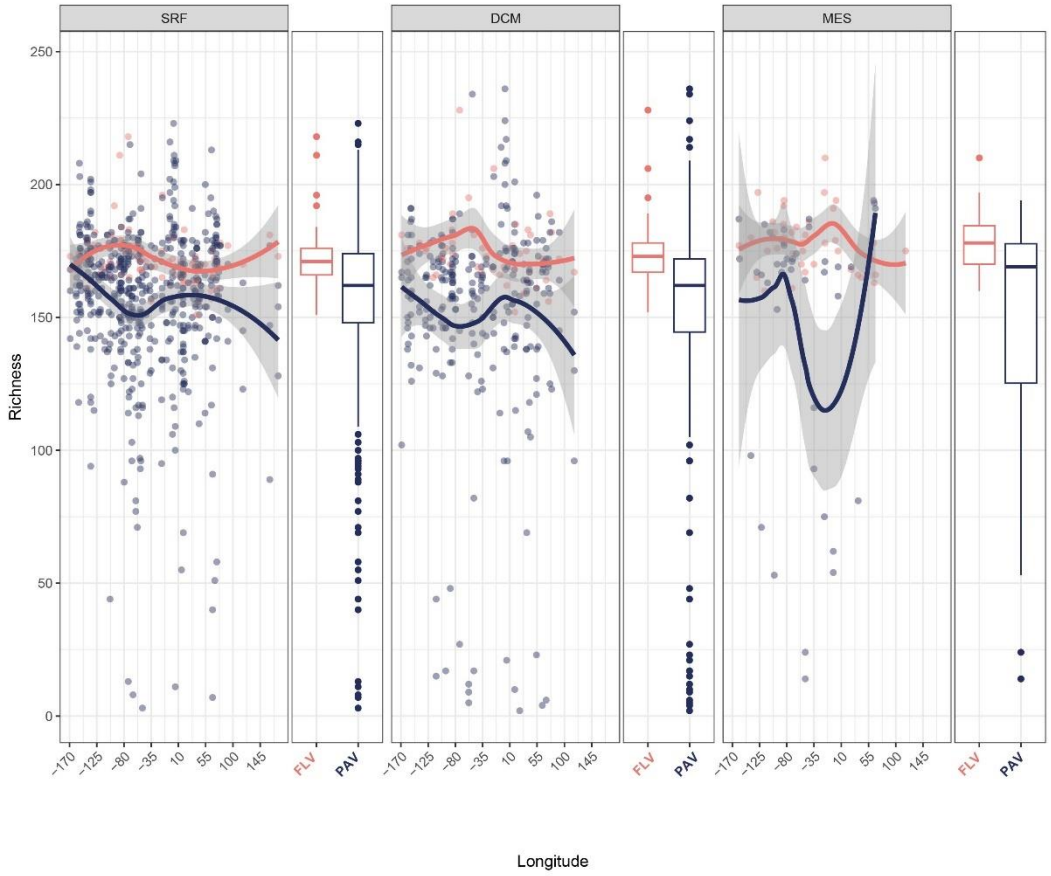

Fig. S1. Vibrio alfa diversity calculated as the richness of Vibrio species across samples and plotted against the longitude of the stations for the different depths: SRF (Surface waters), DCM (Deep Chlorophyll Maximum) and MES (Mesopelagic).

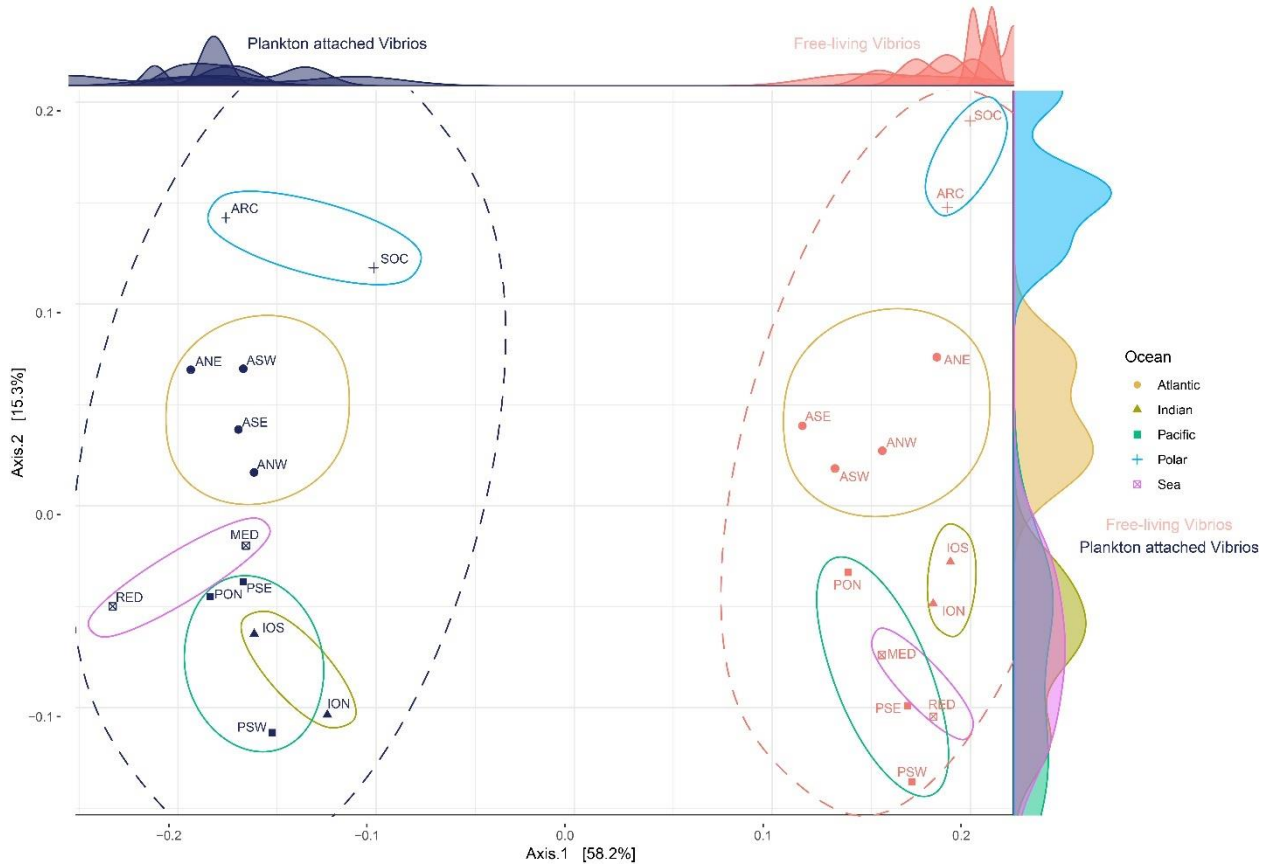

Fig. S2. Beta diversity based on Vibrio taxonomy frequencies (t-PcoA). Dashed ellipses correspond to FLV (freee-livcing Vibrio) (pink) and PLV (plankton attached Vibrio) (blue). Oceanic regions are indicated by the colors of the continuous ellipses and the shapes. The percentage of the variation explained by each axis is indicated in parentheses after the axis label.

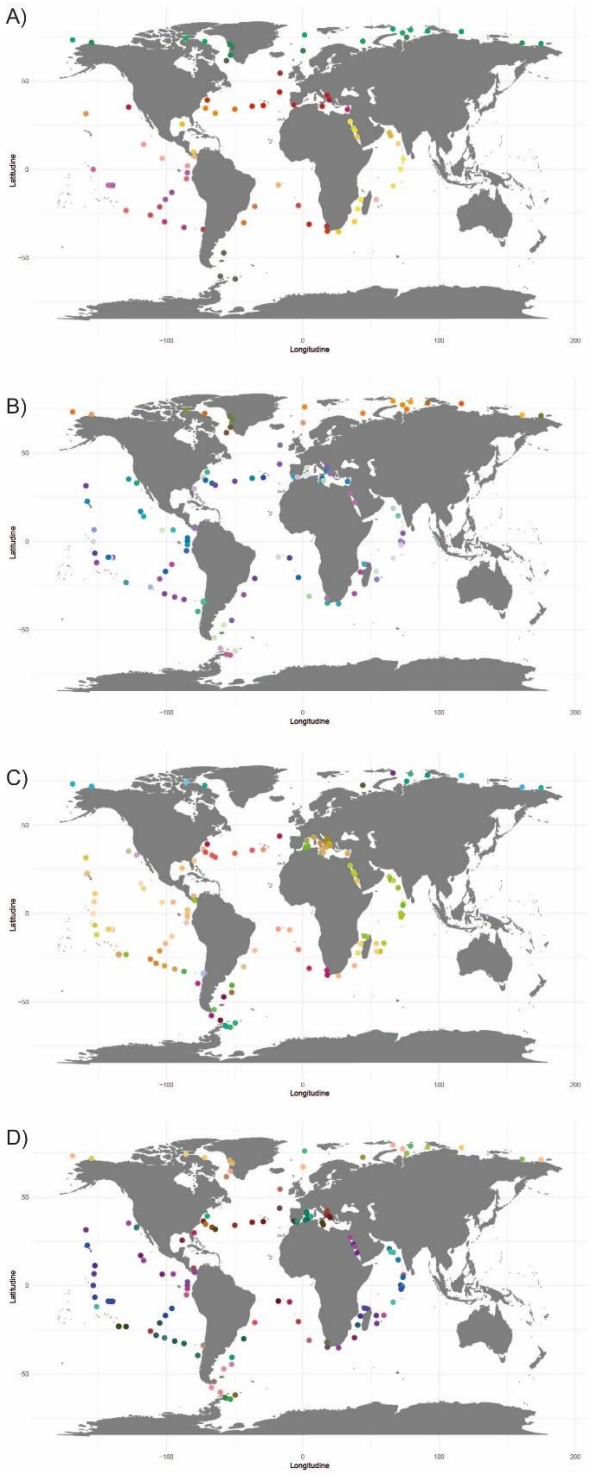

Fig. S3.Genomic provinces of surface stations for the FLV-0.22–3 µm (A), NAV-5-20 µm (B), MiAV-20-180 µm (C) and MeAV-180-2000 µm (D) size fractions. Station colors are derived from the RGB-PCoA based on Vibrio metagenomic dissimilarities, more similar colors indicate more similar communities. Colors can be inconsistent across the different size fractions.

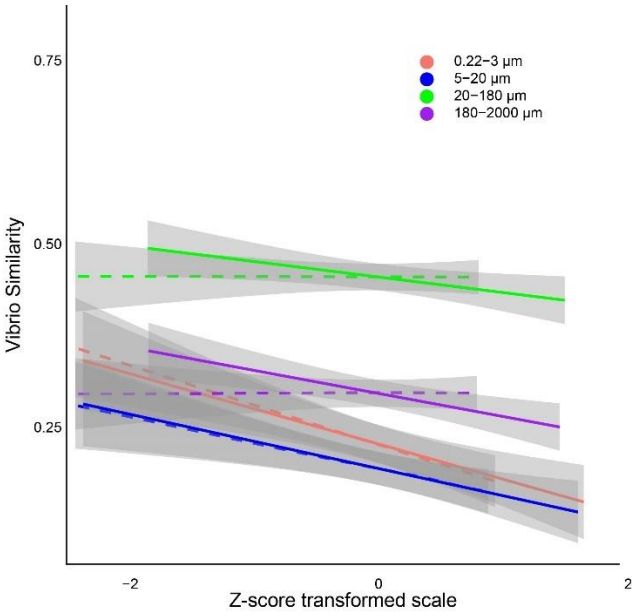

Fig. S4 Comparison between correlation of Vibrio similarity and distance in km (dashed line) and travel time (continuous line). Distance in km was calculated avoiding crossing landing. Correlation coefficients: FLV- 0.22-3 µm (distance km = − 0.38 travel time= -0.33); NAV- 5-20 µm (distance km = − 0.20 travel time= -0.21); MiAV-20-180 µm (distance km = − 0.0015 travel time= − 0.11) ; MeAV 180-2000 µm (distance km = 0.0015 travel time= − 0.17).

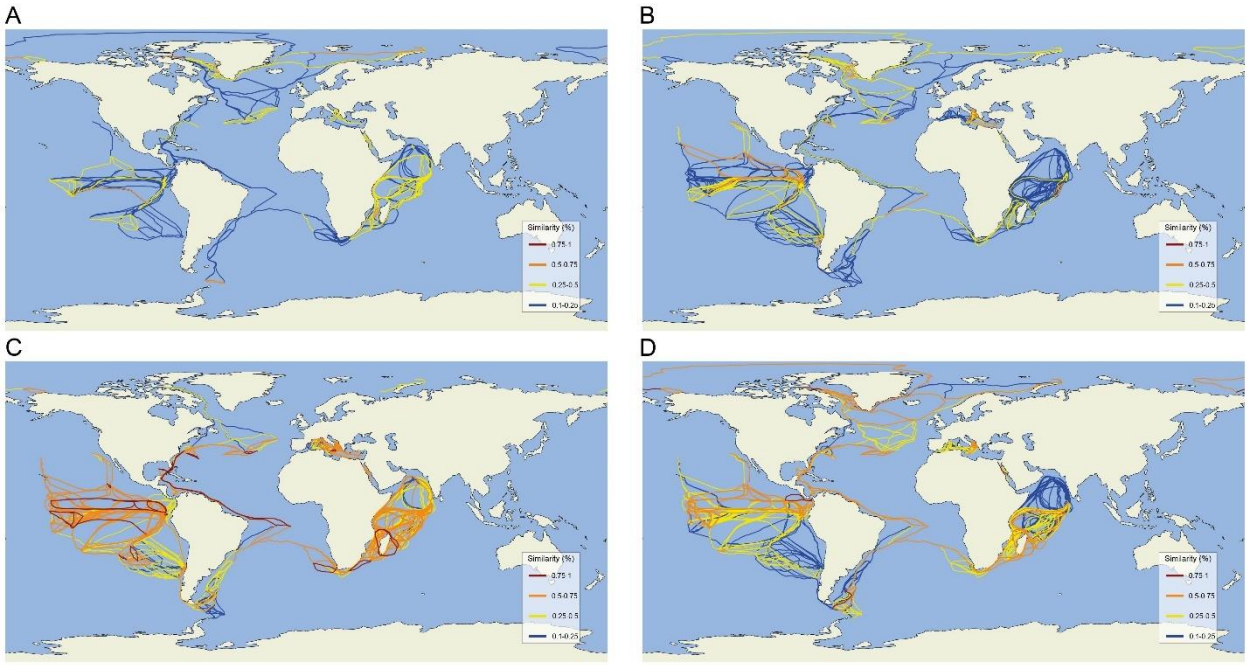

Fig. S5 Networks among stations connected by a travel time less than 1.5 years for the FLV-0.22–3 µm (A), NAV-5-20 µm (B), MiAV-20-180 µm (C) and MeAV-180-2000 µm (D) size fractions. Connections are color-coded based on the similarity of the Vibrio populations between stations.

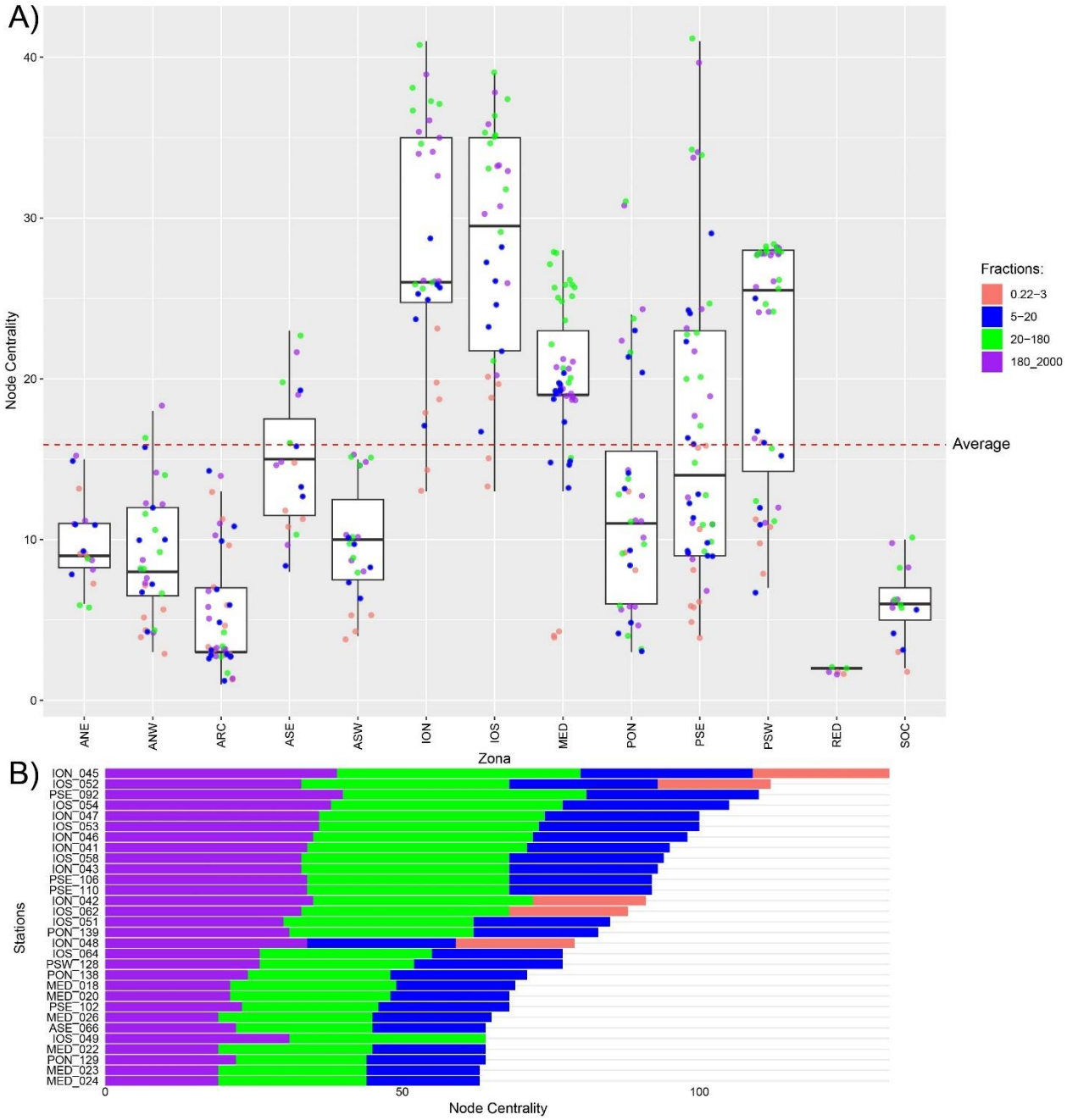

Fig. S6. Node centrality values for Vibrio networks A) Distribution of node centrality for each oceanic region. The average of the node centrality is represented by the red dashed line. B) Top 30 stations with higher values of node centrality. Colors reflect the size fraction in µm.

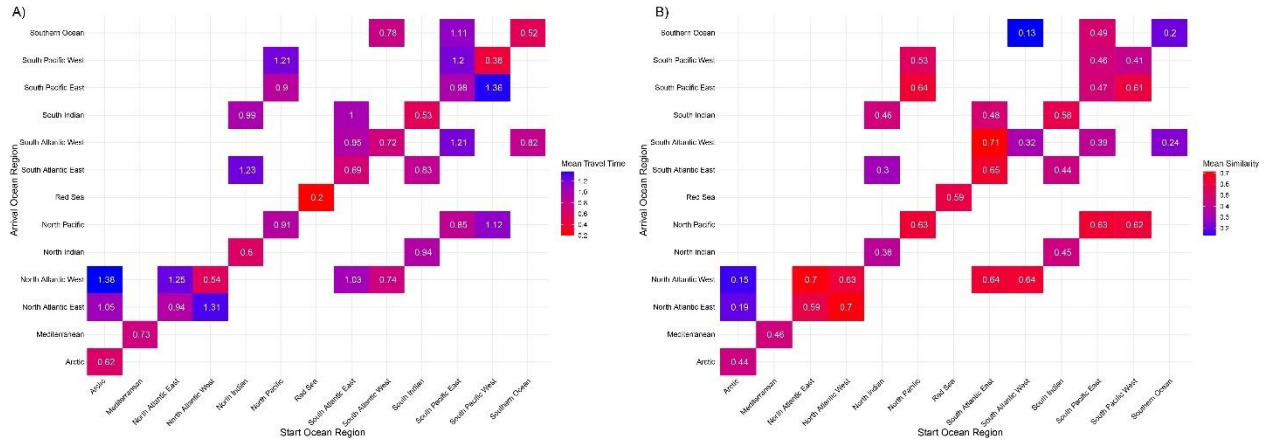

Fig. S7. Average travel time (A) and similarities (B) for the 20-180 µm fraction among sub oceanic regions.

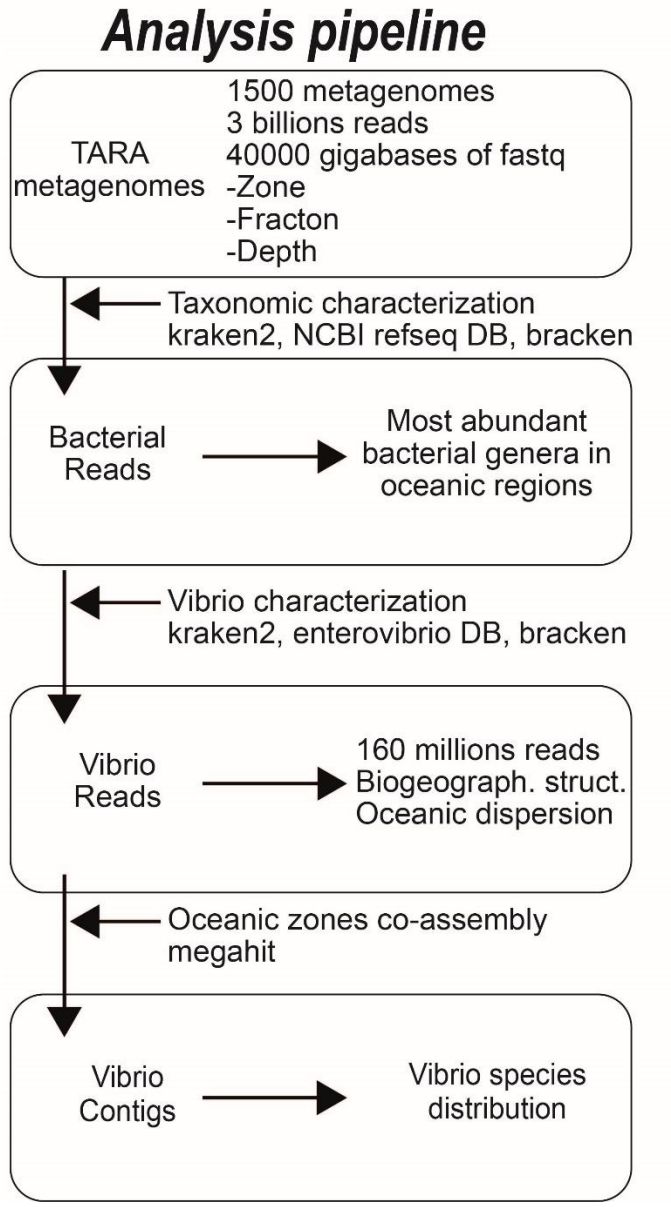

Fig. S8. Brief overview of the pipeline used to analyze the TARA Ocean metagenomes. For the detailed bioinformatic methods see the github repository https://github.com/Luponsky/Deciphering-the-Hidden-Ecology-of-Vibrio-in-the-Oceans

Chapter 4: Large-scale impact of the 2016 Marine Heatwave on the plankton-associated microbial communities of the Great Barrier Reef (Australia)

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Abstract:

The Great Barrier Reef (GBR) is the world's largest coral ecosystem and is threatened by climate change. This study investigated the impact of the 2016 Marine Heatwave (MHW) on plankton associated microbial communities along a ~800 km transect in the GBR. 16S rRNA gene metabarcoding of archived plankton samples collected from November 2014 to August 2016 in this region showed a significant increase in *Planctomycetes* and bacteria belonging to the genus *Vibrio* and *Synechococcus* during and after the heatwave. Notably, Droplet Digital PCR and targeted metagenomic analysis applied on samples collected four months after the MHW event revealed the presence of several potential pathogenic *Vibrio* species previously associated with diseases in aquatic animals. Overall, the 2016 MHW significantly impacted the surface picoplankton community and fostered the spread of potentially pathogenic bacteria across the GBR providing an additional threat for marine biodiversity in this area.

1. Introduction

The Great Barrier Reef (GBR) is the world's largest coral ecosystem, encompassing several thousand reefs along the northeastern coastline of Australia (Pratchett et al., 2018). The GBR is subjected to a range of anthropogenic threats that have resulted in degradation and biodiversity loss in recent decades (De'Ath et al., 2012; GBRMPA, 2017; Hughes et al., 2017, 2018a,b). In particular, summer (January–March) sea surface temperatures (SSTs) in this area have risen substantially over the past century due to global warming (Australian Government Bureau of Meteorology, 2016). Remarkably, in 2016, the GBR experienced one of the warmest temperatures on record, caused by climate change amplified by a strong El Niño (ENSO) from April 2015 to April 2016. Concomitantly, a marine heatwave (MHW) displaying extreme ocean temperatures in February, March and April 2016 (GBRMPA, 2017), leaded to a massive bleaching event (~90% of reefs along the northern region of GBR experienced some bleaching) and resulted in a loss of ~30% of live coral cover over the following six months (Hughes et al., 2017, 2018). Although major ecological changes in biological communities (including macroalgae, fishes and mobile invertebrates) linked to the 2015-2016 ENSO and 2016 MHW events were documented across GBR (Stuart-Smith et al., 2018) little information is available of its impact on marine microbial communities (Hayashida et al., 2020).

Marine microbes are fundamental drivers of biogeochemical cycling and represent the first responders to environmental change that may mitigate or exacerbate the impacts of disturbance for higher trophic levels (Bourne et al., 2016). Studies investigating bacterioplankton community

dynamics across surface-waters of the GBR previously showed that nutrient dynamics linked to anthropogenic impact (e.g. riverine floodwater containing herbicides and excess nutrients from fertilizers) and sea surface temperature (SST) explained the largest amount of inter-seasonal and cross-shelf variation in bacterial assemblages (Angly et al., 2016, Weber et al., 2019, Frade et al. 2020).

MHWs defined as prolonged discrete anomalously warm water events are generally recognized to exert an important impact on ecological and biogeochemical processes in coastal marine environments (Hayashida et al., 2020). In particular, prolonged periods of anomalously high SST during MHWs can rapidly alter the structure and functioning of entire ecosystems. Reduced phytoplankton biomass and weaker phytoplankton blooms were reported during MHWs in oligotrophic waters which are poor in nutrients such as nitrogen and phosphate (Hayashida et al., 2020). The 2011 MHW in western Australian water led also to a substantial decline in zooplankton biomass, abundance and size (Richardson et al, 2020). The poor food environment, which persisted for several months before resetting, could have caused poor feeding conditions and recruitment failures in higher trophic levels (Richardson et al, 2020).

Despite the paucity of data, organisms such as bacteria, with short generation times, might also be significantly affected by MHWs. Of particular concern, bacterial belonging to the *Vibrio* genus includes several species that are pathogenic and represent an important cause of morbidity and mortality in humans and marine animals throughout the world (Ceccareli et al., 2019). These bacteria are indigenous in the marine environment and are mainly found associated with plankton (Baker-Austin et al., 2018). Vibrios tend to be more common in warmer waters, especially above 17°C, and respond rapidly to changes in SSTs, with increasing temperature generally stimulating their spread and pathogenic potential (Vezzulli et al., 2010; Stauder et al., 2010; Trinanes and Martinez-Urtaza, 2021; Kimes et al., 2012; Ushijima et al., 2016).

To investigate the impact of 2015-2016 ENSO and the 2016 MHW on bacterial and *Vibrio* communities in the GBR, this study applied a retrospective molecular analysis of the microbial community associated to archived plankton samples collected by the Australian Continuous Plankton Recorder (AusCPR) survey over an ~800 km transect, from November 2014 to August 2016. Each sample represents five nautical miles of tow (ca. 1.5 m^3 of filtered water) and was previously shown to capture a substantial fraction of the plankton associated bacterial community including the *Vibrio* fraction (Vezzulli et al., 2012, 2022). Overall, the geographic dimension of this study coupled to the large volume of sea surface water (25,000 L) analysed provided first of its kind data

on the impact of the record-breaking 2016 marine heatwave on plankton associated and potential pathogenic bacterial communities in the GBR area.

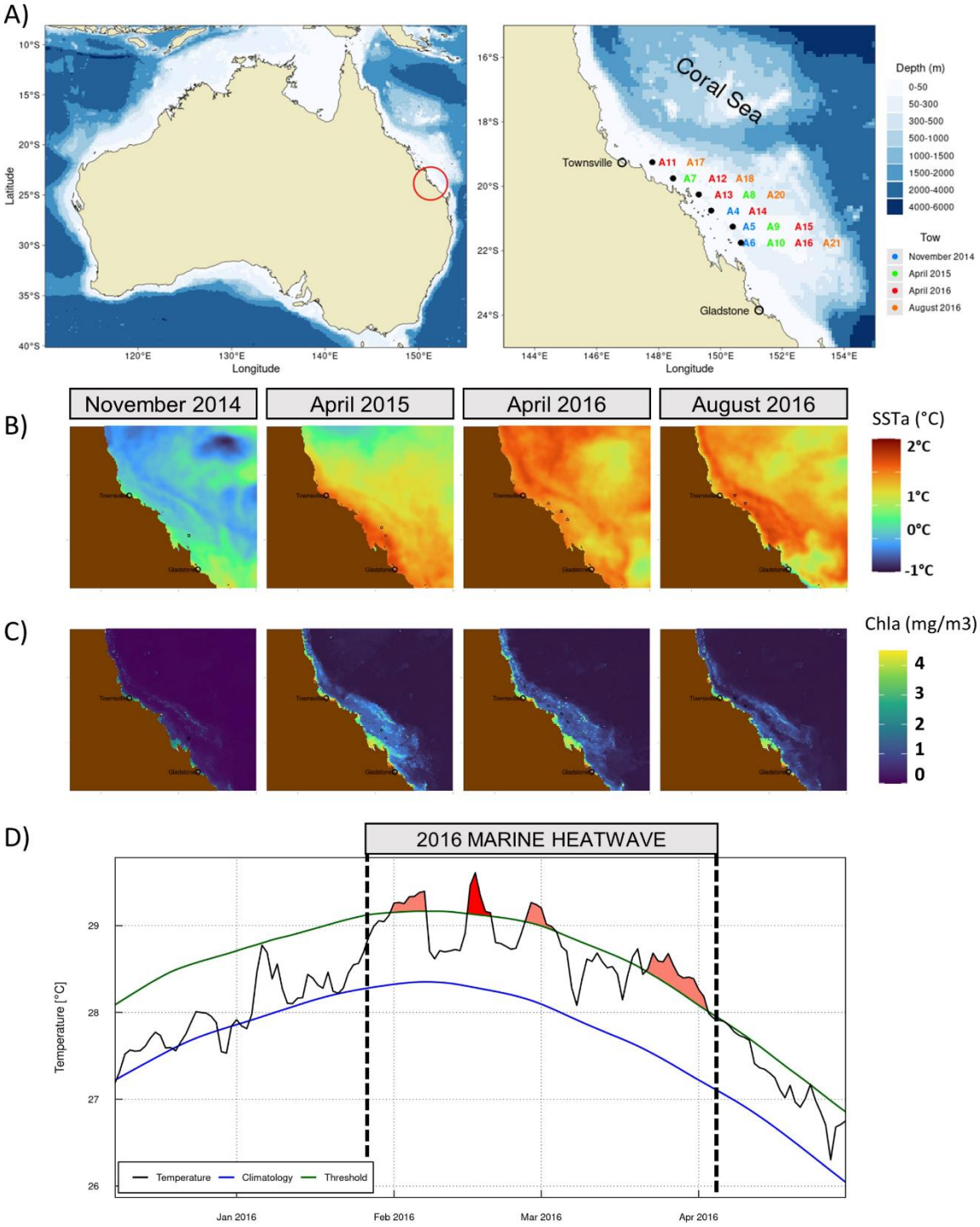

Figure 1 (A) Samples collected in the Great Barrier Reef using the Continuous Plankton Recorder (acronyms refer to sample names). A transect of 800 km was investigated from Gladstone to Townsville during four sampling campaigns in November 2014, April 2015, April 2016 and August 2016 and ca 25,000 L of sea surface water were collected and analysed. Large-scale patterns of environmental variables in north-eastern Australia during the study period plotted as (B) 30 day- average sea surface temperature anomalies and (C) monthly chlorophyll-a concentration. Columns refer to the periods: (1) October 31st–November 29th, 2014 (before the heatwave), (2) March 16th–April 14th, 2015 (at the beginning of the heatwave), (3) March 18th–April 16th, 2016 (during the heatwave) and (4) July 17th–August 16th, 2016 (after the heatwave). (D) 2016 summer MHWs calculated from daily SST time series between Gladstone and Townsville following the methods described by Hobday et al. (2016).

2. Materials and Methods:

2.1 CPR sampling

The Continuous Plankton Recorder (CPR) is a robust mechanical plankton-sampling device towed at 6-10 m depth behind commercial vessels across the globe for more than 80 years (Figure S1) (Reid et al., 2003). Plankton (from bacteria and viruses to copepods and other taxa, particulate organic matter and marine snow are also efficiently captured by the device) enters a small aperture and is captured on a band of silk mesh (270-µm mesh size). The internal mechanism of the CPR advances the silk bands at a constant rate to capture and cover the plankton, which is then wound onto a storage spool in a tank containing formaldehyde (4% final concentration by volume) buffered with sodium tetraborate at pH 7.4 to 7.5. The silk is cut into sections representing 5 nautical miles of tow corresponding to 1.5 m^3 of filtered water.

Seventeen CPR samples collected by the AusCPR survey along an 800 km transect in the GBR were analysed (Figure 1A). Samples were collected by four different CPR tows during the 2015–2016 El Niño in November 2014, April 2015, April 2016 and August 2016. April 2016 was Earth's hottest April, and the period from February-April 2016 was the hottest ever record on the GBR (GBRMPA, 2017) and will be referred to as the 2016 GBR marine heatwave (Hughes et al., 2018).

2.2 Sea Surface Temperatures and Chlorophyll-a data

Sea surface temperature (SST) and sea surface temperature anomaly (SSTa) data were provided by the NOAA Coral Reef Watch (Liu et al., 2014). Temperature data were downloaded using the *heatwaveR* package (Schlegel RW and Smit AJ 2018) and plotted onto the map obtained with *ggOceanMaps* package (Vihtakari 2021) and *ggplot2* package *(*Wickham, 2016). 2016 summer MHWs were calculated from daily SST time series following the methods described by Hobday et al. (2016) for defining heatwaves in marine environments. The R package heatwaveR (Schlegel and Smit, 2018) was used for these calculations. For the purposes, a climatology baseline of a length of 30 years (1987/01/01-2017/01/01) was used, based on the remotely sensed NOAA OISST dataset (Reynolds et al., 2002). Daily OISST data was downloaded for the area between Gladstone and Townsville and averaged for each day. Then, MHWs categories were assessed using the category function based on Hobday et al. (2018) and the result was plotted with the *event_line* function of the heatwaveR package.

Chlorophyll-*a* data, from the Visible Infrared Imaging Radiometer Suite on-board the polar-orbiting Suomi-NPP and NOAA-20 satellites (Wang et al., 2017), was also downloaded and plotted as for the NOAA Coral Reef Watch variables.

2.3 Nucleic acid extraction from CPR samples

For each CPR sample, the filtering silk was cut into five sections (1 x 1 cm^2) and DNA was extracted and purified using the methodology described in Vezzulli et al. (2012, 2016). The amount of DNA extracted from the CPR samples (3-30 ng/µL) was determined fluorimetrically using a QuantiFluor™ dsDNA system with a QuantiFluor™ fluorometer (Promega Italia srl). Sizing of genomic DNA was also conducted in an Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA) using the High Sensitivity DNA kit (Agilent Technologies).

2.4 qPCR

Quantitative Real Time PCR (qPCR) assays were performed to measure the "*Vibrio*-relative abundance index" (VAI) in CPR samples as described by Vezzulli et al. (2012, 2016). VAI is an unbiased molecular index that measures the proportion of *Vibrio* bacteria in relation to total bacteria in CPR samples using qPCR (Vezzulli et al., 2012, 2016). The qPCR amplification protocol was set up on a Light Cycler 1.5 instrument (Roche Diagnostics) using Light Cycler SYBR Green I Master Mix chemistry. The oligonucleotide primers were Vib1 f-5'-GGCGTAAAGCGCATGCAGGT-3' and Vib2 r-5'-GAAATT CTACCCCCCTCTACAG-3' (Thompson et al., 2004), specific for the genus *Vibrio*, and 967f-5'-CAACGCGAAGAACCTTACC-3' and 1046r-5'-CGACAGCCATGCANCACCT-3' (Sogin et al., 2006), specific for the domain "bacteria," amplifying positions 567–680 and 965–1063 (V6 hypervariable region) of the *Escherichia coli* numbering of the 16S rRNA, respectively. Each reaction mixture contained 5.0 mM of MgCl2 and 0.25 µM of each primer in a final volume of 20 µL. The PCR program was as follows: initial denaturation at 95°C for 10 min, subsequent 40 cycles of denaturation at 95°C for 5 s, annealing at 58°C (*Vibrio* spp.) or 57 °C (total bacteria) for 5 s, and elongation at 72°C for 4 s, followed by a final elongation at 72°C for 10 min. For each single real-time PCR assay, each DNA template was analysed in triplicate (coefficient of variation < 5%). *Vibrio* spp. and total bacterial concentrations were expressed as number of cells per square centimeter of CPR sample by dividing total 16S rDNA copy number by the average 16S rDNA copy number in vibrios (n = 9) (Acinas et al., 2004) and Proteobacteria (n = 3.5) (Kormas, 2011), respectively.

2.5 Droplet Digital PCR

The quantification of *V. corallilyticus* was carried out by digital droplet PCR (ddPCR). The ddPCR assays were performed on QX200 Droplet Digital PCR System (Bio-Rad). The reaction mix and droplets preparation (except for the volume of reaction, here 20µl) were already described (Di Cesare et al., 2018). The amplification was performed using a BioRad C1000 Touch Thermal Cycler (Bio-Rad). The amplification conditions were optimized for the *vcpA* gene (Wilson et al., 2013) testing a gradient of annealing temperatures (from 56.2°C to 61°C). After choosing the best annealing temperature (61°C) the final program was set following the manufacture's instruction and the amplification was carried out. Thus, the plates were transferred in the QX200Droplet Reader (Bio-Rad) to analyse the fluorescence signal and to acquire the concentration data. Each DNA template was analysed in duplicate and two no template controls (NTC) and two positive controls (DNA extracted from the strain *V. coralliilyticus* ATCC BAA 450, positive for the vcpA gene) were included in each run. Only reactions with more than 10,000 droplets were analysed, they were between 26,856 and 39,926 (considering two reactions per sample). The threshold used to discriminate between the positive and negative results was manually set considering positive-only samples with a number of droplets ≥ 3 (Di Cesare et al., 2018). Quantification data were analysed by QuantaSoft Analysis Pro software v1.0.596 (Bio-Rad, California) and expressed as gene copy μL^{-1} PCR reaction volume.

2.6 16S rRNA gene sequencing

16S rDNA gene PCR amplicon libraries were generated from genomic DNA extracted from individual CPR samples using primers amplifying positions 965–1063 of the *Escherichia coli* numbering of the 16S rRNA gene that include the V6 hypervariable region (Sogin et al., 2006). Primers were custom designed to include 16S rRNA complementary regions plus the complementary sequences to the Ion Torrent specific adapters. Two PCR assays were performed, a first target enrichment PCR assay with the 16S conserved primers and a second PCR assay, with customized primers including adapters' complementary regions (Table S1). The obtained libraries were sequenced using an Ion Torrent (PGM) Platform (Thermo Fisher Scientific, MA).

Following a similar procedure of Tamburini et al., 2020, raw reads obtained by Ion Torrent sequencing were demultiplexed with SABRE (Joshi, 2013) and quality checked with FastQC (Andrews, 2010), primers were removed with Cutadapt (Martin, 2011). Reads denoising (i.e. trimming and quality filtering) was conducted using Sickle (Joshi and Fass, 2011). Trimmed reads were then imported into the "Quantitative Insights into Microbial Ecology (QIIME 2)" software (v. 2020-11)

(Bolyen et al., 2019) for dereplication and clustering into operational taxonomic units (OTUs). OTUs were picked with *De novo* approach with a default identity of 97%. Chimeras and singletons were identified and removed from the dataset. Taxonomy assignment of representative sequences was done against the Silva database release 138 (Quast et al., 2013) trained with amplification primers to target the V6 region of the 16S rRNA gene.

Statistical analyses were performed using R (R Core Team, 2013) in Rstudio (Rstudio Team, 2015), using Phyloseq package (McMurdie and Holmes, 2013) for biological analysis and GGplot2 for plotting data (Wickham, 2016). Low abundance OTUs < 0.005% (Bokulich et al., 2012) were discarded. The remaining OTUs were normalized by Cumulative Sum Scaling (CSS) transformation, using metagenomeSeq package (Paulson et al., 2013; 2014). Alpha- (i.e., Richness, Shannon and Simpson indices) and beta- (Principal Coordinate Analysis on the Bray–Curtis dissimilarity) diversities were assessed using "*estimate_richness"* and "*ordinate"* functions of the Phyloseq package.

Permutational multivariate analysis of variance (PERMANOVA) was then used to evaluate the null hypothesis that there were no significant differences among tows. PERMANOVA was performed using the *adonis* function in the vegan package (Oksanen et al., 2020) on the Bray–Curtis dissimilarity matrix with 9,999 permutations. The barplot of the most abundant genera was plotted with GGPlot2 package. LEfSe (Linear discriminant analysis Effect Size) was used to determines the organisms most likely to explain differences between different periods by coupling standard tests for statistical significance with additional tests encoding biological consistency and effect relevance (Segata et al., 2011). Sequence reads data were archived at NCBI sequence read archive (SRA) with Accession Number PRJNA811106.

2.7 Target enrichment metagenomics

A target enrichment next-generation sequencing protocol for the analysis of the marine *Vibrio* community was applied on sample A17 (collected after the heatwave and resulting positive for *V. corallilyticus* by ddPCR) following the approach by Lasa et al., 2019. The protocol is based on the use of biotinylated RNA baits (on average >100-mer) for selective capturing of 884 phylogenetic and virulence markers targeting the *Vibrio* community and other potential pathogenic microorganisms in CPR samples via hybridization. Such approach was estimated to increase target DNA coverage by about three orders of magnitude compared with shotgun metagenomics (Vezzulli et al., 2017).

Five hundred nanograms of total RNA baits was produced using the MYcroarray target enrichment proprietary technology (MYcroarray, Ann Arbor, MI) and used for a capture (Vezzulli et al., 2017). Genomic DNA extracted from sample A17 (August 2016), was sized on an Agilent Bioanalyzer and

used for the production of an indexed library for next-generation sequencing on the Illumina platform (Illumina) using the KAPA HyperPlus Kit for Illumina (Roche Diagnostics, Monza, Italy). About 200 ng of the produced library was used for target DNA capturing using the MYbaits protocol (MYcroarray, Ann Arbor, MI) following the manufacturer's instructions. Briefly, the genomic DNA library was heat-denatured and hybridized to the RNA baits in stringent conditions. Hybridization was carried out at 65°C for 36h (capturing of target DNA with 5%–10% sequence divergence is expected at these conditions enabling full covering of marker allelic variants including unknown variants). After hybridization, the biotinylated baits hybridized to captured material were pulled out of the solution with streptavidin-coated magnetic beads and the captured genomic DNA was released by chemical degradation of the RNA baits. Enriched library was amplified and sequenced on a MiSeq Illumina™ platform (V3 flow cell, 600 cycles, 25 M reads 250 bp pair ends).

Raw reads were quality-trimmed using *trimgalore* (Krueger, 2016), merged and chimeras checked with *VSEARCH* (Rognes et al., 2016). Denoised reads went through a pipeline to extract *Vibrio* reads from metagenomes, which consist in a double classification-extraction using, respectively, *kraken2* (Wood et al., 2019) and *extract_kraken_reads.py* from Kraken Tools (Lu, 2018). The first taxonomical extraction was carried out using the Enterobase-*Vibrio* database, a *Vibrio* species genomes curated collection (Zhou et al., 2020). In a second extraction the Refseq Bacteria NCBI database (O'Leary et al., 2016) was used. The extracted *Vibrio* reads were classified at species level aligning them against the refseq prokaryotes database using MetaBlast (Maestri, 2021) using strict alignment parameters (i.e., min_id_perc=85, min_query_cov=70, max_evalue=0.00001). Taxonomy was assigned using the best hit of each blast result.

2.8 Sample contamination

Manipulation of CPR samples for molecular analysis and DNA extraction were carried out in a separate laboratory (nonmarine/nonmicrobiological laboratory) using all necessary precautions (e.g., use of a dedicated set of pipettes, reagents, and consumables to avoid cross contamination of samples).

3. Results

3.1 Changes in Sea Surface Temperature and Chlorophyll-a values in the GBR before, during and after the 2016 MHW

Changes in SSTa patterns over the study period are shown in Figure 1B. Mean monthly SSTa increased from November 2014 (-0.02 ±0.26°C) to April 2015 (0.86 ±0.5°C). Maximum SSTa was recorded in April 2016 (1.22 ±0.22°C) whilst a slightly decrease in SSTa values was observed in August 2016 (1.13 ±0.25°C) *(*Figure 1B). Accordingly, the highest SST value of 29.3°C were recorded during the MHW in April 2016. Monthly mean chlorophyll-a values ranged from 0.14 mg m^{-3} to 0.20 mg m^{-3} over the study period with highest concentration values detected in April 2015 and April 2016 (Figure 1C). Four MHWs events occurred in the study region from February to March 2016 with a duration ranging from 5 to 12 days (Hobday et al., 2016) (Figure 1D).

3.2 Changes in bacterial community composition in the GBR before, during and after the 2016 MHW

A total of 4,002,591 PCR amplicons spanning the V6 hypervariable region of the16SrDNA gene were sequenced from CPR samples collected in this study. Following trimming the number of reads per sample ranged from 32,630 to 251,653 sequences. OTUs number identified by OTUs clustering analysis ranged between 521 (November 2014) to 560 (April 2015) with a mean value of ~500 OTUs per sample (Figure S2A).

No significant difference was found in the bacterial alpha diversity (e.g. Richness, Shannon and Simpson diversity index) among the samples (Kruskal-Wallis rank non-parametric ANOVA, p-value >0.05) (Figure S2A). In addition, rarefaction curves analysis reached a plateau in all samples (Figure S2B), suggesting that the sequencing effort was adequate and detected most abundant phylotypes in the samples.

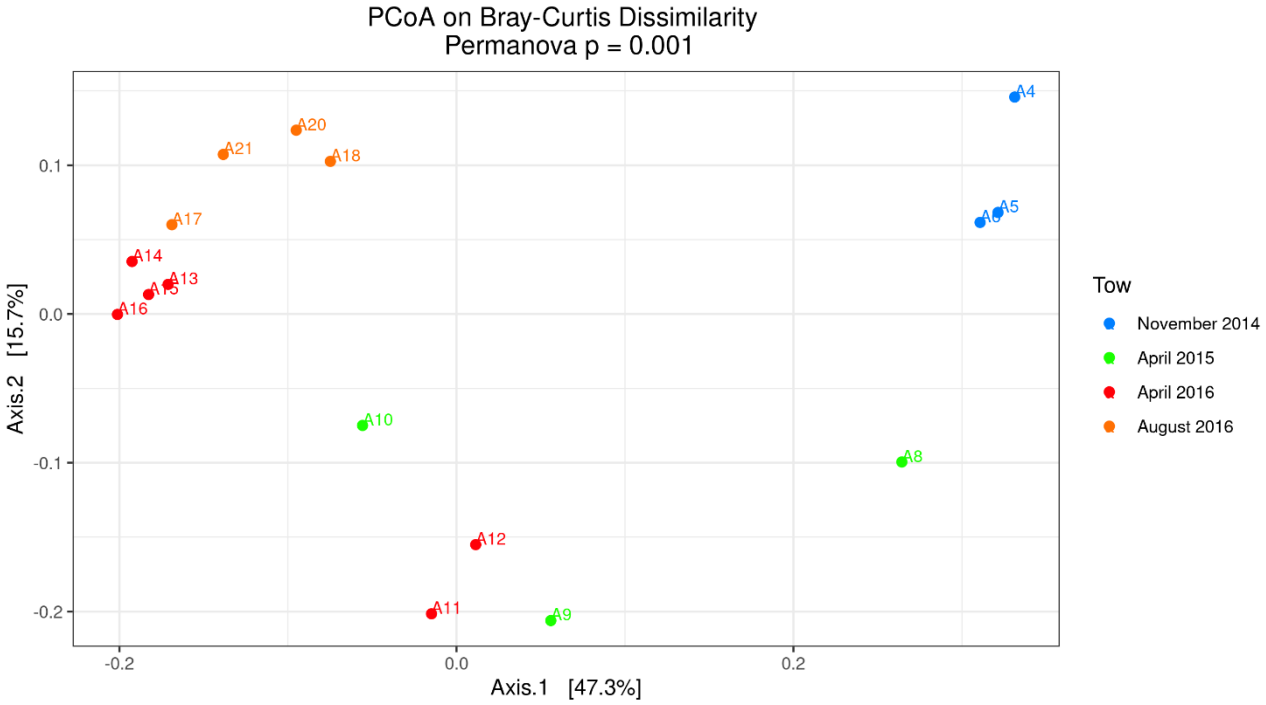

Figure 2 Principal Coordinate Analysis ordination of bacterial communities associated with Continuous Plankton Recorder samples collected in the Great Barrier Reef before, during and after the marine heatwave event in February–April 2016. The percentage of the variation in community structure explained by each axis is indicated in brackets.

The microbial assemblage based on the PCoA clearly separated samples collected before, during and after the heatwave events (PERMANOVA p = 0.001 permutation: 9.999). This suggests that the 2106 MHW was associated to a marked shift in the bacterial community structure. Accordingly, the first two PCoA axes explained 63% of total variance, while the first and third axes explained 61.4% of variation in the bacterial community structure (Figure 2).

Although PCoA can summarize multidimensional clustering of the samples, it cannot identify specific bacterial taxa that are differentially represented (Figure 3A). To this end, discriminant analysis (LDA) effect size (LEfSe) algorithm (Segata et al., 2011) was employed to identify the specific clades that were differentially represented in the samples (Figure 3B). Overall, the most abundant clade was OM190 (Occurrence Frequency of each Genus here defined as the numbers of OTUs assigned to the Genus taxonomic rank-OFG=1025) followed by *Vibrio* (OFG=893), *Synechococcus*_CC9902 (OFG=802), CL500-3 (OFG=712), *Bacillus* (OFG=659) and *Blastopirellula* (OFG=506). Each other clade had an OFG fewer than 500. In particular, a marked increase in the relative abundance of bacteria belonging to the OM190 class and CL500-3 clade (*Planctomycetes)*, *Synechococcus* and *Vibrio* was observed during the MHW event (April 2016). Interestingly, LEfSe results, plotted as a cladogram (Figure 3B) identified *Vibrio* (at different taxonomy ranks) as significantly associated with samples collected in August 2016 following the heatwave event. Accordingly, the OFG of *Vibrio* increased

dramatically during the heatwave in April 2016 (OFG=425) compared to November 2014 (OFG=50) and April 2015 (OFG=109), and remained high during winter in August 2016 (OFG=307) (Figure 3A).

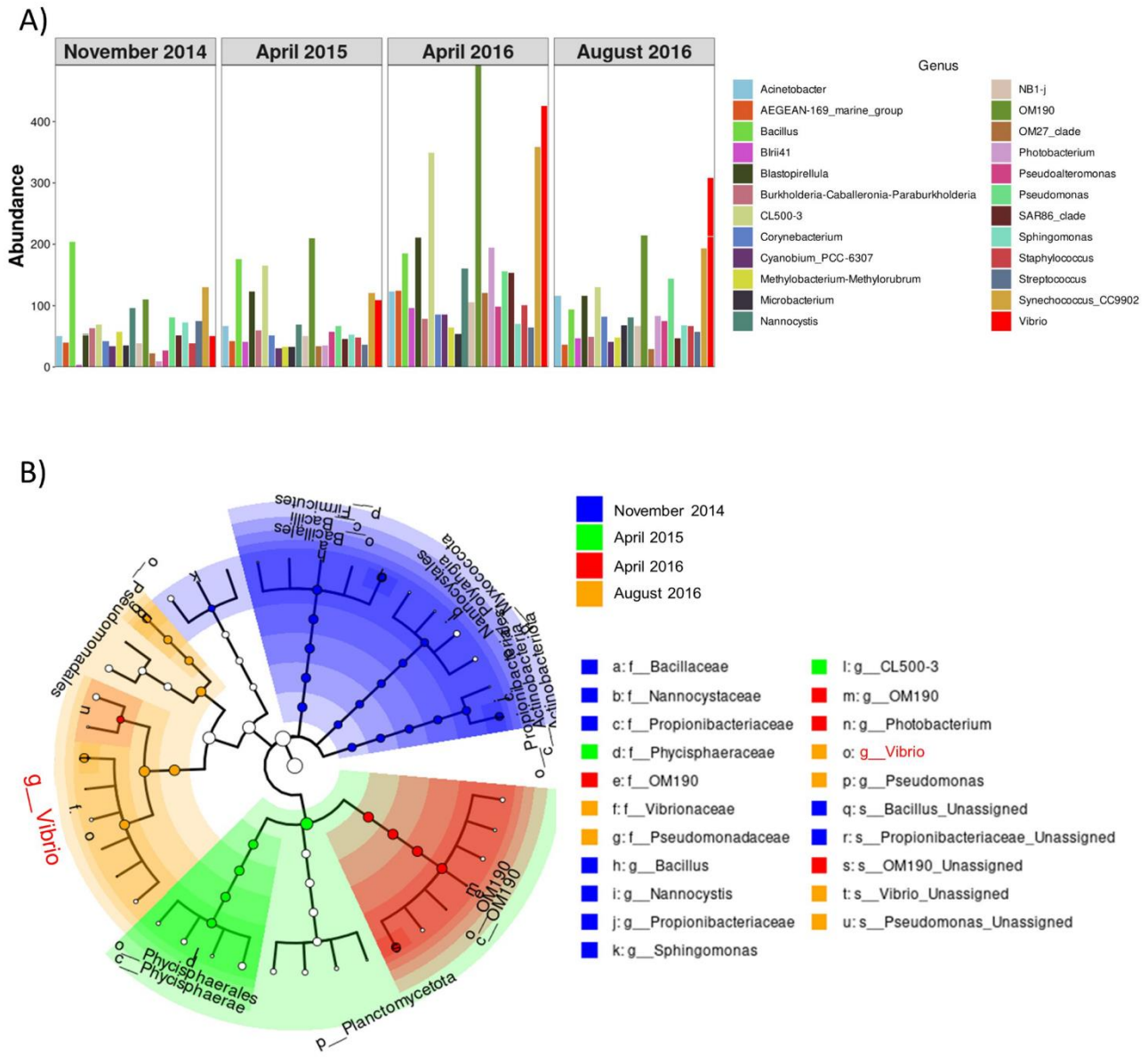

Figure 3 (A) Occurrence Frequency of each bacterial Genus-OFG in the four sampling periods and (B) Cladogram representing results from Linear discriminant analysis Effect Size (LEfSe) comparing different Continuous Plankton Recorder tows (periods) in the Great Barrier Reef. Colors indicate that a statistical association between microbial species and a particular sampling period was detected by the analysis.

3.3 *Vibrio* relative abundance index and potential pathogenic *Vibrio* species

According to sequence data, mean values of *Vibrio* abundance (VAI index) increased significantly during the MHW in CPR samples and remained high in samples collected in August 2016, four months after the MHW event (Figure 4A). Consistently, a positive correlation was found between *Vibrio* OFG and average VAI index (r= 0.99, p-value = 0.003) over the study period (Figure 4B). Although not significant a positive correlation between average VAI index and SSTa (r= 0.84, p-value = 0.16) was also observed (Figure 4C).

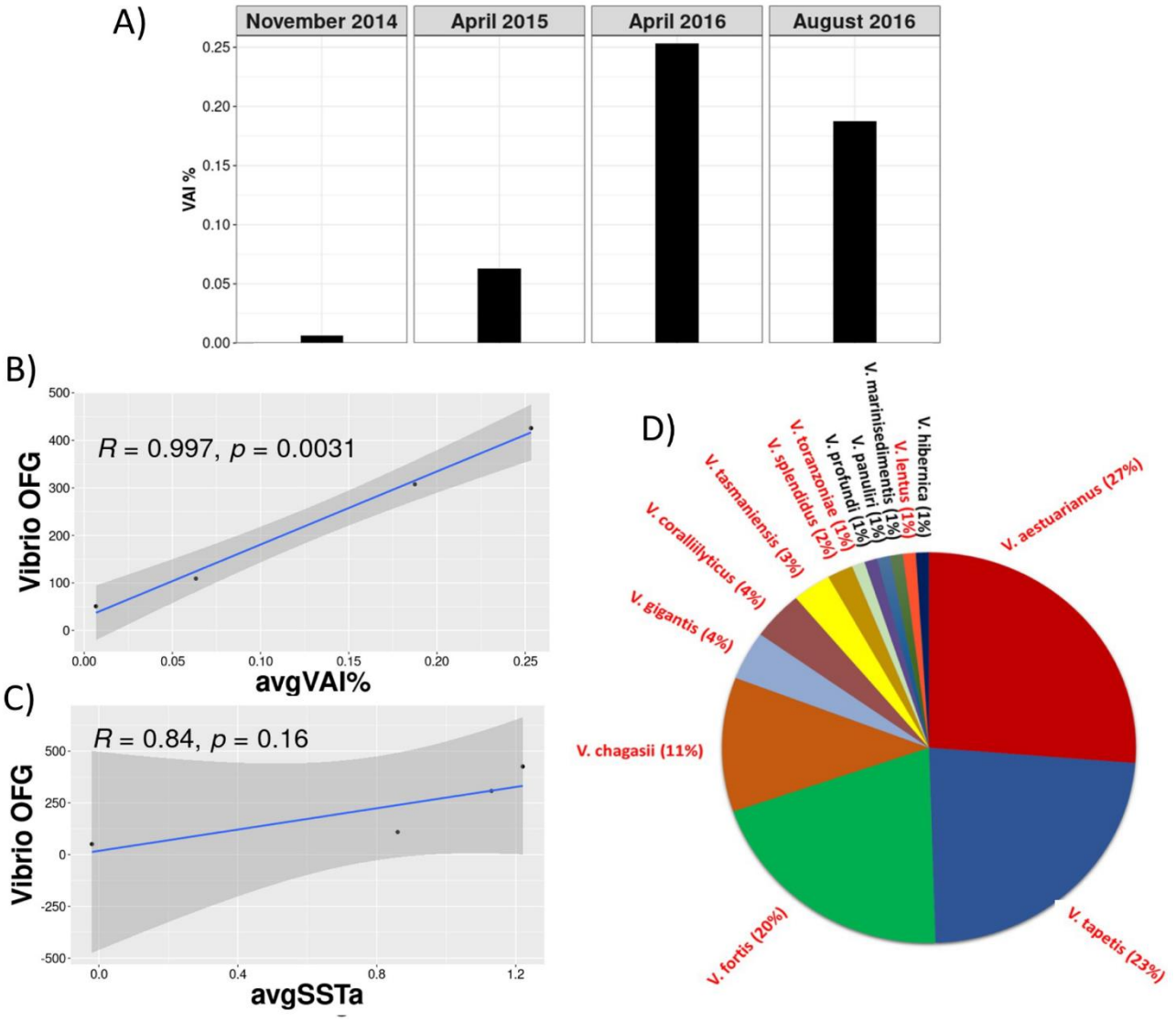

Figure 4 (A) Vibrio relative abundance index-VAI for Continuous Plankton Recorder samples collected in the Great Barrier Reef in November 2014, April 2015, April 2016 and August 2016. Pearson correlation between VAI index and: (B) OFG for the Vibrio genus, (C) SSTa. (D) Occurrence of Vibrio species (%) in sample A17 collected in August 2016, four months after the marine heatwave as assessed by targeted metagenomic analysis (Lasa et al., 2019). Potential pathogenic Vibrio species previously associated with infections in marine organisms are in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Droplet Digital PCR (ddPCR) analysis highlighted the presence of the coral pathogen *V. coralliilyticus* in samples A17 and A21 collected in August 2016 following the MHW. The structure of the *Vibrio* community in sample A17 was further investigated by applying metagenomic analysis targeting 884 phylogenetic and virulence markers of the potential microbial pathogenic community following the protocol described in Lasa *et al*., 2019. Targeted metagenomic analysis revealed the presence of 14 *Vibrio* species in the sample (Figure 4D). According to classification analysis of the sequencing reads, the most abundant species were *Vibrio aestuarianus* (27%), followed by *Vibrio tapetis* (23%), *Vibrio fortis* (20%), *Vibrio chagasii* (11%), *Vibrio gigantis* (4%), *Vibrio corallilyticus* (4%), *Vibrio tasmaniensis* (3%) and *Vibrio splendidus* (2%). Each of the other *Vibrio* species represented <1% of the *Vibrio*

community. Notably, the majority of *Vibrio* species found in sample A17 in August 2016 have been previously associated with diseases in aquatic animals, and thus represent potential marine pathogens (e.g., *V. corallilyticus*, *V. aestuarianus*, *V. tapetis*, *V. fortis*, *V. chagasii*, *Vibrio gigantis*, *V. tasmaniensis*, *V. splendidus*, *Vibrio toranzoniae* and *Vibrio lentus*). Sequences encoding for potential virulence factors such as the M4 family metallopeptidase were also found.

4. Discussion

The impact of MHWs on microbial communities is still poorly understood, despite microbes dominate the ocean's living biomass and play a key role in global biogeochemical cycles (Bar-On and Milo, 2019). In this study, the large-scale impact of the 2016 marine heatwave on plankton associated bacterial communities was investigated along an ~800 km transect in the GBR from November 2014 to August 2016. Four MHWs events occurred in the study region (Gladstone to Townsville) from February to March 2016 with a duration ranging from 5 to 12 days that were classified as moderate based on MHWs categories defined by Hobday et al. (2018) (Figure 1D).

16S rRNA gene sequencing analysis of CPR samples showed a dramatic large-scale shift in GBR picoplankton community structure during the heatwave (April 2016), with observed changes that persisted four months later (August 2016) the MHW event despite a significant drop in SST temperature corresponding to austral winter in the region (Figure 2, 3A). In the study area, SST reached peaks of almost 30°C and daily SST anomaly of ~2°C during the heatwave.

A significant increase in the relative abundance of bacteria belonging to the uncultivated *Planctomycetes* lineages

OM190 and CL500-3 during and after the MHW across the GBR was apparent. *Planctomycetes* are widespread in the marine ecosystem where they have been preferentially detected in the particle-associated microbial fraction (Lage and Bondoso, 2012; Bižić-Ionescu *et al.*, 2015). These bacteria are involved in important processes, such as the mineralization of algal biomass and the removal of nitrogen (Pizzetti et al., 2011). A "*Synechococcus"* bloom was also observed in April 2016 in line with higher chlorophyll-a values detected in this period (Figure 3A,C). Picophytoprokaryotes and especially the cyanobacteria *Prochlorococcus* and *Synechococcus* species are the most abundant primary producers in tropical and subtropical waters of the world ocean, where they generally out-number nano-and microphytoplankton by at least one order of magnitude (Crosbie and Furnas, 2001). Generally, *Prochlorococcus* numerically dominates the phytoplankton community in warm oligotrophic tropical waters whilst *Synechococcus* is distributed further into subtropical zones and is

most abundant in nutrient rich areas (Thomson et al., 2020). *Synechococcus* is also known to forms an important part of the particle-associated microbial community in marine waters (Thiele et al., 2015) which help to explain high relative abundance of this genus found in CPR samples.

Remarkably, bacteria belonging to the genus *Vibrio* were also found to significantly increase during and after the heatwave by both metabarcoding and qPCR analyses (VAI index) of CPR samples. This finding is particularly relevant being *Vibrio* spp. responsible for the majority of human and animal diseases attributed to the natural microbiota of aquatic environments (Baker-Austin et al., 2018). As vibrios are particularly sensitive to fluctuations in seawater temperature at both seasonal (Pfeffer et al., 2003) and long-term (Vezzulli et al., 2016) scales, they have recently been described as a 'barometer' of climate change in the marine environment (Baker-Austin et al., 2017). The observed *Vibrio* expansion in the GBR at a large spatial scale linked with the heatwave supports the view that thermotolerant species showing phenotypic plasticity and better adaptation to thermal extremes such as vibrios could be more resilient to warming events. *Vibrio* has one of the fastest growth rate among bacteria and may thus take advantage over species with lower thermal sensitivity (i.e. less tolerant to heat), possibly occupying the empty niches. Notably, the extent of *Vibrio* expansion across the GBR appears to overwhelm seasonal variations (mostly driven by monthly variations in SST) that, albeit less pronounced in tropical areas, are usually a primary pattern of variability in these bacterial communities. We may speculate that total restoration of the microbial community structure once the pre-heatwave conditions are recovered may be slow to establish, fueling thermotolerant bacteria to persist for longer period of time. Similar changes in microbial community structure linked to MHWs have been reported for coral and bivalve associated microbial communities, where there are also shifts in the animal microbiome structure during periods of thermal stress (Thurber et al., 2009; Green et al., 2019). Again, one of the most consistent changes reported is a sharp increase in bacteria belonging to the genus *Vibrio*. These bacteria are present in the microbiome of healthy animals at low densities, but significantly increase in abundance by up to several order of magnitudes under elevated temperatures (Gibbin et al., 2019; Green et al., 2019).

To assess whether large-scale *Vibrio* expansion following the heatwave was correlated with the proliferation of potential pathogenic species across the GBR, the presence of *V. coralliilyticus* was investigated in CPR samples. *V. coralliilyticus* is known as the aetiological agent of bacterial-bleaching in *Pocillopora damicornis* (Ben-Haim et al 2003), but is also present in other coral diseases, including White Syndrome (Sussman et al., 2008) and Black Band Disease (Arotsker et al., 2009) in the GBR region. By applying an ultrasensitive ddPCR protocol, *V. coralliilyticus* was found in samples collected

in August 2016 following the MHW. To further investigate potential pathogenic bacteria in these samples, a target enrichment next-generation sequencing protocol was applied (Lasa et al., 2019). The protocol is based on the use of biotinylated RNA baits for selective capturing of 884 phylogenetic and virulence markers targeting the *Vibrio* community and other potential pathogenic microorganisms via hybridization (Lasa et al., 2019). Targeted metagenomic analysis of CPR samples using the same protocol was recently applied to retrieve and sequence *V. cholerae* metagenome from a large tropical lake in central Africa (Vezzulli et al., 2021). Taxonomic profiling of the metagenomic sequence reads revealed the presence of several *Vibrio* species including potential pathogenic species (Figure 4D). In addition, sequences coding for proteins related with M4 family metallopeptidase, belonging to *V. coralliitycus*, and *V. aestuarianus* were found. This family comprises metalloproteases described as virulence factors, including *V. coralliilyticus vcpA, vcpB* and *vchA* (De O Santos et al., 2011), suggesting the presence of *Vibrio* pathogenic strains in the sample. Overall, albeit a direct or indirect link between large-scale changes observed in the plankton associated microbial community and human or animal health could not be established, our study suggests that MHWs can foster potential pathogens proliferation in the GBR over a large spatial scale. Warming associated with climate change and exacerbated by MHWs could increase the likelihood of the spread of *Vibrio* pathogens (see also Baker-Austin et al., 2017) which in turn, could pose an additional threat for coral and other susceptible marine organisms (Muller et al., 2008; Miller et al., 2009; Howells et al., 2020).

In conclusion, microbiological analysis of archived CPR samples collected before, during and after the 2016 MHW in the GBR showed a large-scale change in the abundance and structure of the plankton associated microbial communities during the heatwave (April 2016) that persisted over the following months (August 2016). In particular, *Planctomycetes* and bacteria belonging to the genus *Vibrio* and *Synechococcus* showed a marked increase in relative abundance during the heatwave. Furthermore, the spread of potentially pathogenic *Vibrio* species across the GBR highlighted in this study might provide an additional threat for marine biodiversity in this area. Studies investigating MHWs impact on marine microbial communities under a changing climate scenario are thus needed to fully understand how such events could and will affect the health of the GBR ecosystem in the future.

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Chapter 5: General Conclusion

Vibrio are a large group of marine bacteria including several pathogenic species responsible for life-threatening diseases in both human and aquatic organisms. Global change is linked to a worldwide increase in the incidence of *Vibrio*-associated diseases with major impacts on public health and the aquaculture industry worldwide. However, large scale ecological processes governing the distribution and spread of *Vibrio* bacteria in the ocean are still poorly understood. Overall, this thesis explored and illustrated the large-scale patterns, biogeography, and ecology of *Vibrio* species across the world's oceans. The analysis of metagenomic data produced during the TARA Ocean expedition deepened the understanding of *Vibrio* oceanic biogeography, emphasizing the vital role of plankton and currents in its large-scale dispersion through the global ocean. In particular migratory routes defined for plankton can act as a mechanism for the long dispersal of *Vibrio*-attached bacteria across the oceans, which could contribute to recurrent introductions of populations originated in distant areas and contribute to the high diversity and interconnectivity observed in *Vibrio* populations. These processes would have deep implications for shaping the demography, population dynamics and evolution of *Vibrio* species, including those species pathogenic for humans and marine organisms. Meanwhile, retrospective molecular analysis of *Vibrio* populations collected by the Continuous Plankton Recorder survey along a ~800 km transect in the Great Barrier Reef before, during and after a marine heatwave (MHV) revealed that extreme warming events linked with climate change can foster the proliferation of potential *Vibrio* pathogens in the ocean. These findings support the view that thermotolerant microbial species showing phenotypic plasticity and better adaptation to thermal extremes such as vibrios could be more resilient to warming events and possibly take advantage over species with lower thermal sensitivity by occupying their empty niches. Climate change is also changing the routes and speeding up the major ocean currents and altering the biogeographical patterns of zooplankton distributions. As shown in this thesis, all these aspects are intrinsically linked to the dispersal dynamics and connectivity of *Vibrio* populations and govern the microbial community assembly across spatial scales. In consequence, we expect deep changes in the global demography of *Vibrio* populations in the future planet resulting from these changes, with collateral effects on the epidemiology of *Vibrio*-related diseases. Thus, these findings have substantial implications for the ecology of this genus and microbe-plankton interactions, enriching our comprehension of the oceanic environment. Ultimately this work integrated extensive sequencing analysis with environmental data, illustrating the importance of a large and global-scale perspective in understanding *Vibrio*'s biological complexity in relation to plankton, oceanic currents, and climate change.

Publications list

Published:

1. Tamburini, E., Doni, L., Lussu, R., Meloni, F., Cappai, G., Carucci, A., et al. (2020) Impacts of Anthropogenic Pollutants on Benthic Prokaryotic Communities in Mediterranean Touristic Ports. Front Microbiol 11: 1–16.
2. Lasa, A., Auguste, M., Lema, A., Oliveri, C., Borello, A., Doni, L., et al. (2021) A deep-sea bacterium related to coastal marine pathogens. Environ Microbiol 23: 5349–5363.
3. Doni, L., Martinez-Urtaza, J., and Vezzulli, L. (2023) Searching pathogenic bacteria in the rare biosphere of the ocean. Curr Opin Biotechnol 80: 102894.
4. Doni, L., Oliveri, C., Lasa, A., Di Cesare, A., Petrin, S., Martinez-Urtaza, J., et al. (2023) Large-scale impact of the 2016 Marine Heatwave on the plankton-associated microbial communities of the Great Barrier Reef (Australia). Mar Pollut Bull 188: 114685.
5. Doni, L., Tassistro, G., Oliveri, C., Balbi, T., Auguste, M., Pallavicini, A., et al. (2023) Plankton and marine aggregates as transmission vectors for V. aestuarianus 02/041 infecting the pacific oyster Crassostrea gigas. Environ Microbiol Rep 15: 631–641.
6. Auguste, M., Leonessi, M., Balbi, T., Doni, L., Oliveri, C., Vezzulli, L., and Canesi, L. (2024) Seasonal fluctuations of hemolymph microbiota and immune parameters in Mytilus galloprovincialis farmed at La Spezia, Italy. Aquaculture 578: 740028.
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9. E. Bosi, E. Taviani, A. Avesani, L. Doni, M. Auguste, C. Oliveri, M. Leonessi, J. Martinez-Urtaza, C. Vetriani, L. Vezzulli (2024) Pan-genome provides insights into Vibrio evolution and adaptation to deep-sea hydrothermal vents. Genome Biology and Evolution
10. M. Auguste, M. Leonessi, L. Doni, C. Oliveri, A. Jemec Kokalj, D. Drobne, L. Vezzulli, L. Canesi (2024) Polyester microfibers exposure modulates Mytilus hemolymph microbiome. International Journal of Molecular Sciences

Submitted for publication:

1. Deciphering the Hidden Ecology of *Vibrio* in the Oceans (chapter 3 of this thesis), Under review in Science Advances

In preparation:

1. Milestones in *Vibrio* history: From Ancient Origins to Modern times (chapter 1A of this thesis).
2. Short and long reads metabarcoding from a complete North Atlantic transect.
3. dadaWRAP, a one-line completely automated metabarcoding pipeline.
4. First screening of microbial assemblages in corals from Maldives atolls.

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