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Tomás Dias Araújo

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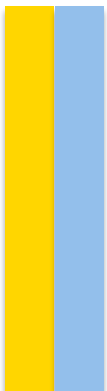

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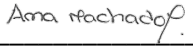

Ana Machado

À minha querida avó Graça

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For now, what is important is not finding the answer, but looking for it.

From *Gödel, Escher, Bach, an Eternal Golden Braid*, Douglas R. Hofstadter

Resumo

O uso recreativo da água balnear tem vindo a aumentar, trazendo consigo benefícios sociais e económicos, com um impacto positivo no lazer, turismo, comércio e promoção da saúde. No entanto, esta crescente interação entre Homem-água apresenta também potenciais riscos à saúde. O contacto direto com a água balnear pode expor o ser humano a diversas bactérias patogénicas, incluindo aquelas naturalmente presentes nos sistemas aquáticos, podendo potencialmente conduzir a doença. As bactérias do género *Vibrio* são reconhecidas como agentes patogénicos emergentes para os humanos, podendo causar gastroenterites, infeções de pele, respiratórias e sépsis. De facto, o número de vibrioses associadas ao uso recreativo de água balnear têm vindo a aumentar no Hemisfério Norte, possivelmente consequência das alterações climáticas em curso. No entanto, a Diretiva Europeia referente ao uso da Água Balnear (2006/7/EC) considera apenas a poluição fecal para estimar o risco para a saúde pública. O estudo aqui apresentado pretendeu explorar a prevalência simultânea de bactérias indicadoras de contaminação fecal (FIB) e bactérias autóctones potencialmente patogénicas, de forma a avaliar a robustez dos critérios legais para garantir a segurança dos banhistas. Foram recolhidas amostras mensais de água superficial em dez praias atlânticas (NW de Portugal) durante um período de 13 meses, cobrindo pois, uma época balnear. A enumeração das FIB foi realizada por contagem em placa de acordo com as normas ISO e a abundância de *Vibrio* spp. e *Vibrio parahaemolyticus* foi estimada pelo método do número mais provável-reação em cadeia da polimerase (MPN-PCR). A identificação presuntiva dos isolados de *V. parahaemolyticus* foi efetuada através de meios de cultura seletivos e diferenciais, seguida de confirmação por PCR. Os isolados confirmados como *V. parahaemolyticus* foram caracterizados para avaliação da diversidade, assim como dos perfis de virulência e resistência antimicrobiana. Durante a época balnear, a qualidade da água cumpriu os critérios legais europeus determinados para a prática balnear. Tendo em conta o período de estudo em apreço, foi possível verificar uma associação positiva entre os níveis de contaminação fecal e a precipitação, provavelmente devido ao potencial *run-off* terrestre ou incapacidade de tratamento das águas residuais. Os *Vibrio* spp. foram detectados durante todo o período do estudo, com abundâncias até 4,40 x 10^5 MPN / mL. Foi possível identificar uma tendência sazonal com abundância superior de *V. parahaemolyticus* totais ((<LOD (Limite de Detecção) - 4,40 x 10^5 MPN / mL)) e enteropatogénicos *tdh*+ e *trh*+ (< 4,40 MPN / mL) durante os meses mais quentes, coincidindo com a época balnear. Tendo em conta a origem distinta dos dois grupos de bactérias, observou-se uma correlação significativa negativa ($p < 0,05$) entre o *V. parahaemolyticus* e as FIB. A salinidade, temperatura e Chlα demonstraram desempenhar um papel-chave na dinâmica do *V. parahaemolyticus*, estando positivamente associados com abundâncias superiores. Dos 66 isolados confirmados como *V*. *parahaemolyticus*, 18%, 12% e 3% exibiram o gene associado com a virulência que codifica a hemolisina termolábil (*tlh*), a hemolisina direta termoestável (*tdh*) e a hemolisina direta termoestável relacionada (*trh*), respetivamente. Apesar de se encontrarem na mesma área geográfica, os isolados de *V. parahaemolyticus* exibiram ampla diversidade genética, formando dois grupos de acordo com o perfil de genes de virulência. A suscetibilidade antimicrobiana dos isolados potencialmente

patogénicos foi avaliada usando o método de difusão em disco de Kirby-Bauer, tendo revelado padrões de resistência preocupantes, particularmente a β-lactâmicos, aminoglicosídeos e macrólidos, sendo que todos os isolados revelaram multirresistência. Este estudo destaca a relevância da monitorização contínua de *Vibrio* spp. em águas balneares, especialmente no contexto das alterações climáticas, assim como a importância da abordagem *One Health* na construção e coordenação das estratégias de saúde pública para garantir a segurança dos banhistas.

Palavras-chave: *Vibrio parahaemolyticus*, *Vibrio* spp., Indicadores de contaminação fecal, Águas balneares, Ambiente e Saúde Pública, *One Health*

Abstract

Recreational water use has been increasing with social and economic benefits, positively impacting leisure, tourism, trade, and health promotion. However, this increased human-water interaction also presents potential health risks. Direct contact with water can expose individuals to a variety of pathogenic bacteria, including those naturally present in the water, potentially leading to waterborne diseases. *Vibrio* spp. are recognized as emerging pathogens to humans, known to cause diarrheal illnesses, wounds, skin and respiratory infections, and septicemia. Indeed, vibriosis outbreaks associated with recreational bathing are on the rise in the Northern Hemisphere linked to the ongoing climate changes. However, the current European Bathing Water Directive (2006/7/EC) only considers fecal pollution. The aim of the study was to investigate fecal indicator bacteria (FIB), along with naturally occurring potential pathogenic bacteria, to assess the robustness of the legal criteria for safeguarding the health of beachgoers. Monthly water samples were collected from ten Atlantic beaches (NW Portugal) over a 13-month period. FIB enumeration was performed by plate count following ISO guidelines. *Vibrio* spp. and *Vibrio parahaemolyticus* abundance were estimated by the most probable number-polymerase chain reaction (MPN-PCR). Selective differential media were used for presumptive identification of *V. parahaemolyticus* isolates, followed by PCR confirmation and assessment of diversity, virulence, and antibiotic resistance profiles. Overall, during the bathing season FIB complied with European legal criteria. However, higher levels were associated with precipitation events. *Vibrio* spp. were successfully detected throughout the study period, with abundances up to 4.40 x 10^5 MPN / mL. A seasonal trend was observed, with higher total *V. parahaemolyticus* (<LOD - 4.40 x 10^5 MPN / mL), and enteropathogenic *tdh*+ and *trh*+ *V. parahaemolyticus* (< 4.40 MPN / mL) abundances found during the warmer months, coinciding with the bathing season. *V. parahaemolyticus* were significantly ($p < 0.05$) negatively correlated with FIB. Salinity, temperature, and Chl*a* appear to play a key role in *V. parahaemolyticus* dynamics, being associated with higher abundances. Of the 66 confirmed *V. parahaemolyticus* isolates, *tlh* gene encoding the thermolabile hemolysin was found in 18%, whereas 12 and 3% exhibited the thermostable direct hemolysin (*tdh*) and thermostable-related direct hemolysin (*trh*), respectively. Despite being within the same geographic area, the *V. parahaemolyticus* isolates displayed a wide genetic diversity, clustering according to the virulence gene profile. Antibiotic susceptibility testing using the Kirby-Bauer disc diffusion method revealed concerning resistance patterns, particularly to β-lactam, aminoglycosides and macrolide antibiotics, with all the potentially pathogenic isolates exhibiting multidrug resistance. This study highlights the relevance of continuous monitoring of *Vibrio* spp. in recreational waters, especially in the context of climate change, and the importance of the One Health approach to effectively manage public health strategies and ensure the safety of beachgoers.

Keywords: *Vibrio parahaemolyticus*, *Vibrio* spp., FIB, Recreational water, Environment and Public Health, One Health

Abbreviation list | Lista de abreviaturas

AMR – Multiple antibiotic resistance

APA - Agência Portuguesa do Ambiente

APW – Alkaline Peptone Water

BWD – Bathing Water Directive

CAV – CHROMagar Vibrio

CFU – Colony Forming Unit

Chl α – Chlorophyll α

CLSI – Clinical and Laboratorial Standards Institute

dNTP – Deoxynucleotide

EDTA – Ethylenediaminetetraacetic acid

ERIC-PCR - Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction

EUCAST – European Committee on Antimicrobial Susceptibility Testing

FC – Fecal Coliforms

FIB – Fecal Indicator Bacteria

IE – Intestinal Enterococci

ISO - International Organization for Standardization

LB – Luria Bertani

LOD – Limit of Detection

MAM-7 – Multivalent Adhesion Molecule 7

MPN-PCR - Most Probable Number-Polymerase Chain Reaction

POM –Particulate Organic Matter

PCR – Polymerase Chain Reaction

PM – Particulate Matter

SST – Sea Surface Temperature

TAE – Tris-Acetate-EDTA

TCBS – Thiosulfate-Citrate-Bile Salts-Sucrose

TDH – Thermostable Direct Hemolysin

TLH – Thermolabile Hemolysin

TRH – TDH-Related Hemolysin

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Declaration of contributors | Declaração de contribuidores

The work presented in this dissertation was performed by the author under the supervision of Professor Doutor Adriano Bordalo e Sá, PhD, and Ana Machado. The author also played an active and substantial role in sampling, experimental procedures, and results analysis, which were essential for the preparation of this document.

Introduction | Introdução

Recreational water can pose public health risks due to the diverse microorganisms comprising the bathing water microbiome.[1] These microorganisms can be introduced through anthropogenic activity, such as fecal matter, or be naturally habitants of the aquatic ecosystems with potential to cause disease.[1] Coastal regions play a critical role, not only in the local, regional, and national economies, but also contributing to the wellbeing and social and cultural activities of the communities.[2] However, direct exposure to microorganisms is promoted during occupational or recreational aquatic activities in these areas, reason why bathing waters are considered prime places of infection.[3] Consequently, the quality of bathing waters must be recognized as a public health concern.

The first European regulation for bathing waters was published in 1975, when the Bathing Water Directive (BWD) (76/160/EEC) was created, and was later revised in 2006 (2006/7/EC). According to the latter BWD, the water quality assessment and estimation of health risk for users is determined by two Fecal Indicator Bacteria (FIB), *Escherichia coli* and *Enterococcus* spp..[4] Recently, it has been suggested that additional monitoring and implementation of preventative measures for naturally occurring microorganisms, such as *Vibrio* spp. and cyanobacteria, should be considered, as they could be even of higher importance than fecal pollution.[1,3,5] However, the current legal criteria dates from 2006.

Current climate warming is unequivocal, with evidence of increased global air and ocean temperatures, widespread melting of snow and ice, and rising global average sea level. Global sea surface temperatures (SST) have risen by approximately 0.88 °C since 1970 and are expected to keep rising.[6] Among other consequences, changes in climate can have a negative impact on water security, lead to a marine microbial community change with ideal environmental conditions to pathogens grow and spread to new ecological niches, and consequently foster waterborne diseases.[6,7]

The genus *Vibrio* comprises 146 species (http://www.bacterio.net/vibrio.html)[8], of which at least 12 are known to cause human infections.[9] The majority of human infections linked to the natural microbiota of aquatic habitats and seafood are caused by *Vibrio*.[10,11] From these, *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus,* and *V. alginolyticus* were the most relevant human pathogenic species, also known as the "big four".[12] Human infections caused by vibrios can be categorized as cholera and non-cholera infections, denominate as vibriosis.[10] *V. cholerae* is a major contributor to disease in low-income countries, where access to safe water and proper sanitation are lacking, while *V. parahaemolyticus* and *V. vulnificus* infections are typically associated with consumption of raw or undercooked seafood or exposure (skin wound or water ingestion) during fishing or recreational swimming in the developed world.[9,10] Inhalation of contaminated aerosol has also been reported as transmission route to *V. parahaemolyticus*.[1]

Vibrios are gram-negative, halophilic, rod-shaped bacteria autochthonous in freshwater, estuarine and marine environments.[9] Several studies have demonstrated that vibrios constitute a substantial portion of the ocean biomass, making up over 10% of the bacteria that can be cultured from water samples[13], depending on the specific geographic region and environmental conditions. Vibrios prefer warm, particularly above 17 °C, brackish water, and alkaline conditions.[12] Several studies have now shown that the key determinants for *Vibrio* spp. prevalence in natural habitats are temperature and salinity.[14] Vibrios demonstrate a broad spectrum of niche adaptations, existing in the aquatic environment as free floating organisms or by attachment to biotic (e.g. copepods, crustacean, cyanobacteria, algae), and abiotic surfaces.[9,15] Moreover, persistence in the environment when environmental conditions are unfavorable, can be facilitated by the ability to assume survival forms, including biofilm formation, and a "viable but nonculturable" (VBNC) state.[16,17] In this state, bacterial cells become metabolically dormant and difficult to isolate in culture media, while maintaining virulence proprieties.[9]

V. parahaemolyticus was first described in Japan in 1950 by Tsunesaburo Fujino as the causative agent of food borne disease outbreak, which recorded 272 cases of diarrheal disease with 20 deaths after consumption of semi-dried fish.[18] Infections were geographically restricted until the emergence of the O3:K6 pandemic clone 1996.[19] Subsequently, the highly pathogenic ST36 *V. parahaemolyticus* clonal type emerged in the United States[20], and has undergone geographic expansion.[10]

Similarly to other *Vibrio* species, *V. parahaemolyticus* is found in estuarine, marine and coastal environments, in a planktonic state or attached to zooplankton, fish, crustaceans, as well as to inert surfaces.[10,21] The prevalence *of V. parahaemolyticus* is known to be associated to environmental conditions, namely SST, salinity, pH, chlorophyll *a*, and turbidity, with optimal survival and growth conditions at salinity around 30, and temperature within the range of 20 to 35 °C.[22,23] These bacteria are characterized by a remarkable rapid growth, with a replication time of 8 to 9 min when under optimal conditions.[9]

Despite the limited epidemiological surveillance, it is acknowledged that *V. parahaemolyticus* infections are increasing worldwide linked to climate change, including previously low-incidence areas like South America and Europe (Spain, France, United Kingdom, northern European countries).[9,24-26] Furthermore, *V. parahaemolyticus* is also recognized as the leading cause of human gastroenteritis associated with seafood consumption worldwide.[26] Foodborne transmission accounts for an estimated 80% of *V. parahaemolyticus* infections in the US[27], and is an important cause of seafood-borne illness in many Asian countries[28-31]. Non-foodborne transmission routes, primarily exposure to recreational water, were responsible for 17% of the reported infections in the US[27]. Mirroring the prevalence in the aquatic environment, *Vibrio* spp. infections show a marked seasonal distribution, primarily occurring during warmer months.[32]

The ability of the microbiota to cause disease and its severity depends on the route of exposure, inoculum size, virulence traits, and host attributes, such as immune system impairment and/or underlying diseases.[10,12,33] The infectious dose for *V. parahaemolyticus* is estimated between 10^5 – 10^7 cells[9,34,35], however, the ST36 clonal type demonstrates pathogenicity at lower infectious doses (10^3 – 10^4 cells).[20]

Acute gastroenteritis caused by *V. parahaemolyticus* is typically self-limiting and does not require medical intervention. Symptoms include watery diarrhea, abdominal cramps, nausea, vomiting, fever and headache and usually occur within 24 h of ingestion lasting for 1 to 12 days. Rehydration is the primary treatment, but occasionally hospitalization may be necessary[9,26], and high fevers and/or patient with underlying disease can need antibiotic therapy.[10] Wound infections, however, frequently need medical intervention with appropriate antibiotic therapy and even surgical debridement.[10,12] Complications of wound infections cases happen especially in patients with underlying diseases and/or immunocompromised[1,10], and can evolve to pathologies such as cellulitis, necrotizing fasciitis, and sepsis.[36-38]

Clinically isolated strains of *V.* parahaemolyticus are strongly associated with β-hemolytic activity on Wagatsuma blood agar, known as Kanagawa phenomenon. Major virulent factors of *V. parahaemolyticus* include the pathogenic toxins such as thermostable direct hemolysin (TDH), responsible for the Kanagawa phenomenon, TDH-related hemolysin (TRH), and thermolabile hemolysin (TLH), encoded by *tdh*, *trh* and *tlh* genes, respectively. Expression of *tdh* and *trh* are considered the most predictive overall indicators of virulence.[9,10]

V. parahaemolyticus pathogenesis begins with the attachment to host cells, facilitated by specific adhesion factors, particularly the Multivalent Adhesion Molecule 7 (MAM-7). MAM-7 is a key adhesin playing a crucial role in the initial stages of infection.[9,39] The protein interacts with the phosphatidic acid and the fibronectin on host cell surfaces, ensuring that the bacteria remain anchored to the host, and establishing a foothold for subsequent infection phases. Following attachment, *V. parahaemolyticus* employs several toxins and secretion systems to invade and damage host tissues. TDH and TRH exert hemolytic, enterotoxic, cardiotoxic and cytotoxic effects by binding to and disrupting cell membranes. Additionally, *V. parahaemolyticus* employs two type III secretion systems (T3SS1 and T3SS2) to mediate the translocation of effector proteins into host cells.[39,40] T3SS1 is conserved in all strains inducing cytotoxicity, whereas T3SS2 is identified primarily in pathogenic strains causing enterotoxicity and helping in immune evasion.[41] Effector proteins associated with T3SS2 like VopQ, VopS, and VopA interfere with host cell signaling and cytoskeletal structures, promoting autophagy and cell death.[39]

Portugal has a coastline of c.a. 990 km and 620 km^2 of inland waters. About 85% of the population inhabits the coastal region (European average of 42%), where most of the larger cities are located, rising further up in the touristic summer months.[42] Furthermore, with a total of 664 designated

bathing waters in 2024[43], Portugal accounts for about 3% of the European Union classified bathing areas.[44]

Addressing the concern of potential rise of vibriosis and adopting the integrated One Health approach, the main objectives of this study were: (a) ascertain if the official criteria for bathing water classification based solely on FIB were sufficient to ensure the safeguard of users; (b) investigate the prevalence of *Vibrio* spp, particularly *V. parahaemolyticus*, in recreational waters; (c) explore the link between environmental constraints and the prevalence of potential pathogenic bacteria (FIB and *Vibrio* spp.); (d) characterization of *V. parahaemolyticus* isolates virulence and antibiotic profile to understand the associated public health implications.

Materials and Methods | Materiais e Métodos

Study design and sample collection

Surface water samples were monthly collected at ebb tide from 10 beaches on the Northwest of Portugal (Atlantic Ocean), over a 13-month period (**Figure 1**). The exact location of each beach was obtained by means of GPS (Magellan 600, Magellan). The samples were collected in 0.5 L sterile plastic bottles, kept refrigerated in the dark, and processed within 4 h of collection. In the northern coastal Portuguese beaches, the official bathing season for 2018 spanned between June, 15 and September, 18.

Analytical procedures

Temperature, conductivity, turbidity, dissolved oxygen, oxygen saturation, and pH were measured *in situ*, using a multiparameter probe (YSI 6920, YSI Incorporated).
Precipitation (mm) data were obtained from APA (Agência Portuguesa do Ambiente).[45]
For the determination of total particulate matter (PM), and organic particulate matter (POM), 0.5 L water samples were filtered through pre-combusted glass filters (GF/F Whatman), and dried at 105 °C (PM), followed by combustion at 500 °C, 2h, according to APHA *et al.,* 1992.[46]
Chlorophyll *a* (Chl*a*) concentration was determined spectrophotometrically according to Parsons *et al.*, 1984[47], after extraction using 90% acetone.
Ammonium concentration was determined colorimetrically using the method described in Grasshoff *et al.*, 1983.[48]

Fecal indicator bacteria (FIB)

Samples for fecal indicator bacteria evaluation were filtered onto sterile gridded cellulose nitrate membranes (0.45 µm pore size, 47 mm diameter, Whatman), and placed on mFC-agar (Difco) and Slanetz-Bartley agar (Oxoid) plates for enumeration of thermotolerant fecal coliforms (FC) and

intestinal enterococci (IE), respectively. Incubation was performed at 44.5 °C for 24 h (FC), or 48 h (IE).[49] Typical colonies were counted, and results expressed as colony forming units (CFU) / 100 mL.

For water quality classification, FIB levels were interpreted according the European Directive 2006/7/CE[4], transposed into the Portuguese national law by Decree-Law No. 135/2009 of November 03[50], and the threshold limits (1000 CFU / 100 mL for *E. coli* and 300 CFU / 100 mL for EI) establish by the Technical Monitoring Committee on May 2020 for single samples assessment.[45]

Quantification of *Vibrio* spp. and *V. parahaemolyticus*

To quantify the *Vibrio* spp., total and enteropathogenic *V. parahaemolyticus* abundance in the coastal recreational water samples, a combined most probable number-polymerase chain reaction (MPN-PCR) method was applied.[51] Water samples (300 mL), were concentrated onto membrane filters (0.22 µm pore size, 0.47 mm diameter, Whatman), and incubated in alkaline peptone water (APW) at 35 °C for 4 h for bacteria recovery. After enrichment, nucleic acid was extracted from 1 mL of APW by boiling according to Ausubel *et al.* 1990.[52] Briefly, aliquot was centrifuged for 10 min at 13,200 rpm. The supernatant was discarded, the pellet was resuspended in 1 mL of nuclease free water, and incubated 15 min at 100 °C. The suspension was then centrifuged at 2,000 rpm for 3 min, the supernatant transferred into a new sterile tube, and used as template for PCR.

Primer sequence, expected amplicon size, annealing temperature, and extension time are given in **Table.** PCR reactions were performed using Promega GoTaq Green Master Mix (Promega, United States of America) in 25 µL reactions containing 20 pmol/µL of each primer and 5 µL of DNA template (undiluted, 10-fold dilutions until negative signal was achieved).[53,54] All PCR amplifications were carried out in an C1000 Thermal Cycler (Biorad, USA) set to cycling conditions as follows— initial denaturation at 94 °C for 10 min, followed by 35 cycles of denaturing at 94 °C for 30 s, annealing temperature (°C) for 30 s, and 72 °C for time extension (min) according to the **Table**, and final extension at 72 °C for 10 min. PCR products were separated by agarose gel (1.5%) electrophoresis in 1X TAE buffer (0.04 mol/L Tris-acetate, 0.001 mol/L EDTA [pH 8.0], stained with GreenSafe (Nzytech, Portugal), and visualized under UV light. MPN values were calculated from statistical tables of Man 1983[55], and expressed as MPN per mL.

Isolation and characterization of *V. parahaemolyticus*

The isolation of *Vibrio* spp. was done according to Huq *et al.*[56] procedures. Triplicates water samples of 500 mL were filtered through sterile nitrocellulose membranes (0.2 µm pore size, 47 mm diameter, Whatman, United Kingdom). Membranes containing seston were placed directly on thiosulphate-citrate-bile-salts-sucrose (TCBS, Liofilchem, Italy), and CHROMagar Vibrio (CAV, CHROMagar, France) agar plates and incubated for 24 h at 37 °C. Presumptive *Vibrio* spp. colonies

were isolated and subculture from the selective media onto Luria Bertani Broth (LB, Fluka, United Kingdom) and stored at –80 °C in LB broth with 25% glycerol until further analysis.

The isolates stored at -80 °C were cultured in 1.5 mL of LB broth and incubated for 18 h at 37 °C with shaking at 150 rpm. Bacterial pellets were recovered by centrifugation at 13,000 rpm for 10 min at room temperature. Each pellet was resuspended in 500 µL of sterile distilled water and boiled for 10 min at 100 °C, after which the samples were allowed to cool until room temperature, and then centrifuged at 2,000 rpm for 3 min. The supernatant was used as template for PCR reactions.

Vibrio spp. genus confirmation was performed by PCR using the 567F/680R *Vibrio*-specific primer set.[57] *ToxR* gene for PCR targeting was considered for *V. parahaemolyticus* isolates identification according to Okuda *et al.,* 1999.[58] DNA extraction of each isolate was performed by boiling method previously described[52], as described above. Details including primer sequences, expected amplicon sizes, annealing temperatures, and extension times are given in Subsequent PCR targeting virulence factors, *tlh*, *trh* and *tdh,* were performed on all the confirmed *V. parahemolyticus* isolates according to Bej *et al.,* 1999[59] in 50 µL multiplex PCR reactions containing 1 µM of each primer and 5 µL template DNA. Thermal cycling conditions were as follows: initial denaturation at 94 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 60 s, 58 °C for 60 s, extension at 72 °C for 60 s, and final extension for 10 min at 72 °C.[59] Details for all PCR amplifications, including primer sequences, expected amplicon sizes, annealing temperatures, and extension times are given in the **Table**.

PCR products were separated by agarose gel (1.5%) electrophoresis (120 V for 60 min), in 1X TAE buffer (0.04 mol/L Tris-acetate, 0.001 mol/L EDTA [pH 8.0], stained with GreenSafe (Nzytech, Portugal), and visualized under UV light.[58] Hypperladder IV (Bioline, London, UK) was used as molecular weight marker.

<u>*V. parahaemolyticus* phylogeny</u>

V. parahaemolyticus isolates carrying the virulence factors *tlh*, *trh*, *tdh* were proceeded to a long-range Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR). The procedure followed essentially Chokesajjawatee *et al.*, 2008[60] to determination of clonality and relatedness.

Amplification reactions were carried out in 50 µL volumes, using the Takara *Ex Taq* DNA polymerase and buffer system (Takara Mirus Bio Corporation, Madison, WI). The final PCR mixture comprised 5uL *Ex Taq* buffer (with 2 mM $MgCl_2$), 4 µL of 200 µM of each deoxynucleotide (dNTP), 2 µL of 800 nM of each ERIC primer, 1.25 units of *Ex Taq* DNA polymerase and 5 µL of DNA template. Primer

sequences used were the universal ERIC primers, as follows: ERIC-1, 5'-ATG TAA GCT CCT GGG GAT TCA C-3'; and ERIC-2, 5'-AAG TAA GTG ACT GGG GTG AGC G-3'.[61]

The PCR cycle program included an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 52 °C for 1 min, and extension at 65 °C for 10 min and a final extension step at 65 °C for 20 min. PCR products were separated by agarose gel (1.5%) electrophoresis (80 V for 5 h and 30 min), using a 1 mm comb, in 1X TAE buffer (0.04 mol/L Tris-acetate, 0.001 mol/L EDTA [pH 8.0], stained with GreenSafe (Nzytech, Portugal), and visualized under UV light. Hypperladder IV (Bioline, London, UK) was used as molecular weight marker.

V. parahaemolyticus antibiotic susceptibility

Antibiotic susceptibility assessment was carried using the Kirby-Bauer disc diffusion method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines[62], and in the absence of recommendation, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[63] To ensure a standardized bacterial concentration for testing, the inoculum density was adjusted to 0.5 McFarland turbidity standard before spreading onto Mueller-Hinton agar plates with the help of a sterile swab. Antibiotic discs were placed on the surface of the inoculated media and the plates were incubated at 37 °C for 18 h.

The isolates were tested against 20 antibiotics (Oxoid) belonging to 9 different classes: Penicillins – Penicillin 10 µg (PEN10), Ampicillin 10 µg (AMP10); Cephalosporins – Cefoxitin 30 µg (FOX30), Cefotaxime 30 µg (CTX30), Ceftriaxone 30 µg (CRO30), Ceftazidime 30 µg (CAZ30), Cefepime 30 µg (FEP30); Carbapenems – Imipenem 10 µg (IMP10); Monobactams – Aztreonam 30 µg (ATM30); Aminoglycosides – Gentamicin 10 µg (GMN10), Kanamycin 30 µg (KNM30), Streptomycin 10 µg (SMN); Tetracyclines - Tetracyclin 30 µg (TET30), Doxyciclin 30 µg (DOX30); Fluoroquinolones – Ciprofloxacin 5 µg (CIP5), Nalidixic acid 30 µg (NAL30); Macrolides – Erythromycin (ERY15) and Miscellaneous agents – Chloramphenicol 30 µg (CHL30), Trimethoprim – Sulfamethoxazole 25 µg (SXT25), and Rifampicin 5 µg (RIF5). The inhibition zone diameter was measured and interpreted according to the EUCAST[62] and CLSI[63] guidelines as resistant, intermediate, or susceptive. *Enterobacteriaceae* guidelines were used whenever official guidelines for *Vibrio* were non-existent. The strain *Vibrio parahaemolyticus* ATCC 17802 was used as quality control.

Statistical Analysis

Abundance data exploration and analysis were carried in R version 4.4.0 R Core.[64] The data were firstly explored following the protocol described by Zuur *et al.,* 2010.[65] Furthermore, data were tested for normality using the Kolmogorov-Smirnov test and for homoscedasticity using Leven test.[66] Due to the lack of data normality, the Spearman's rank correlation coefficient was used to

assess the relationship of environmental factors and microbiological indicators abundance. Concerning the correlation coefficient, the subscript letters stand for the specific parameter. The significance level used was 0.05. The fingerprint patterns from the *V. parahaemolyticus* ERIC-PCR gels were analyzed using the computer software package Quantity one 1-D analysis software version 4.6.9 (Bio-Rad). A presence or absence matrix was generated and exported to the Primer 6 software package (version 6.1.11; Clarke and Warwick)[67], sand used as input data to evaluate similarity between isolates. Hierarchical clustering analysis following the group average method with the default parameters (5 % significance, mean number of permutations 1,000, number of simulations 999) was performed using the Bray–Curtis similarity method.

Results | Resultados

Fecal indicators

Microbiological analysis revealed that overall, FIB levels met the Portuguese and European legal criteria throughout the bathing season (**Figure 2**), enabling a classification of "*excellent*" for bathing. Thermotolerant fecal coliforms levels ranged from 0 to 48,153 CFU / 100 mL, while intestinal enterococci levels were at least one magnitude order lower for the same sample, varying between 0 and 24,077 CFU / 100 mL. FC and IE were significantly ($r = 0.87$, $p < 0.05$) correlated, as expected (**Figure 3**). Significant ($p < 0.05$) positive correlations were observed between both fecal indicators and precipitation ($r_{FC} = 0.49$, $r_{IE} = 0.47$), turbidity ($r_{FC} = 0.19$, $r_{IE} = 0.26$), and ammonium ($r_{FC} = 0.44$, $r_{IE} = 0.37$). Contrary, FIB exhibited ($p < 0.05$) significant negative correlations with temperature ($r_{FC} = -0.28$, $r_{IE} = -0.23$), and salinity ($r_{FC} = -0.53$, $r_{IE} = -0.44$). To note that increased precipitation was linked to higher turbidity ($r = 0.25$), particulate organic matter ($r = 0.17$), and ammonium concentration ($r = 0.27$), and lower salinity ($r = -0.45$).

Vibrio spp. and *V. parahaemolyticus* abundance

Vibrio spp. were successfully detected throughout the period of study, with an average abundance of 4.12×10^5 MPN / mL ($1.90 \times 10^4 – 4.40 \times 10^5$ MPN / mL). No significant ($p > 0.05$) difference between months was detected (**Figure 4**).

Total *V. parahaemolyticus* were detected in the recreational waters at levels ranging from below the limit of detection (LOD) to 4.40×10^5 MPN / mL, whereas enteropathogenic *tdh*+ and *trh*+ *V. parahaemolyticus* were found at lower levels, up to 4.40 MPN / mL. A seasonal trend was observed, with total *V. parahaemolyticus* abundance and toxigenic genes prevalence significantly ($p < 0.05$) higher during the warmer months, coinciding with the bathing season (**Figure 5**).

Total *V. parahaemolyticus* and enteropathogenic *tdh*+ and *trh*+ *V. parahaemolyticus* showed positive correlation with each other, but were significantly ($p < 0.05$) negatively correlated with FIB (**Figure 3**). Significant ($p < 0.05$) negative correlations were observed between precipitation and total *V. parahaemolyticus* ($r = -0.29$), *trh*+ *V. parahaemolyticus* ($r = -0.30$), and *tdh*+ *V. parahaemolyticus* ($r = -0.19$). Higher salinity was associated with increased levels of total *V. parahaemolyticus* ($r = 0.21$), and *trh*+ *V. parahaemolyticus* ($r = 0.18$), but only temperature showed a significant ($p < 0.05$) positive correlation with *tdh*+ *V. parahaemolyticus* ($r = 0.39$). The prevalence of virulence associated genes in *V. parahaemolyticus* was negative correlated with turbidity ($r_{trh} = -0.34$, $r_{tdh} = -0.26$), particulate matter ($r = -0.26$), and ammonium concentration ($r = -0.25$), although not significantly ($p > 0.05$). Additionally, a significant ($p < 0.05$) positive correlation was found between total *V. parahaemolyticus* and Chl*a* concentration ($r = -0.29$).

<u>*V. parahaemolyticus* identification and characterization</u>

From a library of colonies generated after presumptive identification on selective differential media, 643 isolates were identified as belonging to the genus *Vibrio* using PCR, and 66 isolates were further identified as *V. parahaemolyticus.* Out of the 66 confirmed *V. parahaemolyticus* isolates, 20 (approximately 30%) carried at least one of the virulence associated genes. The *tlh* gene encoding the thermolabile hemolysin was successful detected in 12 isolates (18%), whereas 8 (12%) and 2 (3%) isolates exhibited the thermostable direct hemolysin and thermostable-related direct hemolysin, respectively.

The antibiotic susceptibility of a subset (13 isolates) of enteropathogenic *tdh*+ and *trh*+ *V. parahaemolyticus* was evaluated to further characterize their pathogenic profile (**Figure 6**). All the isolates showed susceptibility to gentamicin, doxycycline, trimethoprim – sulfamethoxazole, ciprofloxacin, imipenem, and ceftazidime. Contrarily, 100% of the isolates showed resistance to penicillin. Resistance was also observed in 92% of isolates for ampicillin, 85% for rifampicin, and 77% for erythromycin. Notably, 2 isolates exhibited at least intermediate resistance to second and third generation cephalosporins - cefotaxime and cefuroxime, 2 isolates showed intermediate resistance to tetracycline, 1 isolate showed intermediate resistance to chloramphenicol and one isolate displayed resistance to ciprofloxacin. Moreover, all the isolates were classified as multidrug resistant, meaning that resistance to at least one antibiotic from three or more different classes was observed. Antibiotics from the β-lactam, aminoglycosides, and macrolide classes revealed the highest resistance rates.

Although retrieved from the same geographic area (NW Portugal), the *V. parahaemolyticus* isolates displayed a wide genetic diversity, evidenced by the low degree of genetic similarity, with only 20% resemblance observed between some isolates (**Figure 6**). Notwithstanding, two distinct groups emerged and could be identified, each harboring different virulence genes.

Discussion | Discussão

Recreational waters users are susceptible to exposure to a diverse range of microorganisms, including naturally occurring pathogens, such as *Vibrio* spp.. Rising water temperatures and changing precipitation patterns may have a major impact on the microbiota of coastal water systems, by increasing the occurrence of autochthonous bacteria and enhancing anthropogenic inputs, such as FIB to coastal waters.[7,68]

The results of this study corroborated that even when FIB levels met the BWD standards, classifying beaches as "excellent" for bathing, it may not be sufficient to safeguard public health, because they deal with fecal contamination, only. Indeed, the need to reevaluate the water quality assessment criteria have been proposed by other studies, and even recognized by the World Health Organization.[1,5,69,70] In this study, FIB and *Vibrio* spp., particularly *V. parahaemolyticus* were found to have a negative correlation, demonstrating a distinct origin. Higher FIB levels were positively correlated with precipitation, turbidity, and ammonium concentration, indicating that rain events can lead to increased run-off and sewage overflow, carrying contaminants from land to coastal waters.[68] In contrast, *Vibrio* spp. exhibited a positive correlation with salinity, consistent with their marine origin.

The recognized ubiquity of *Vibrio* spp. in the aquatic environment corroborated the detection found throughout the year in this study, uncovering the potential for year-round exposure to these potentially pathogenic bacteria. The association between warmer months, salinity, and Chl*a* with the prevalence of *V. parahaemolyticus* as previously described in different geographic locations.[22,23,71,72] While the values require cautious interpretation due to the limitations of the MPN-PCR method (a probabilistic quantification technique), this study revealed higher abundances of *Vibrio* spp. and *V. parahaemolyticus* compared to the majority of previously reported studies[5,14,53], suggesting a potentially ecological niche adaptation, and in line with the geographical transition proposed in association to climate change.[24,72] The infectious dose of *Vibrio* spp. varies among species and specific strains, and with the transmission route. For instance, the minimum infectious dose of *V. cholerae* via water consumption is estimated to range between 10^4 - 10^6 cells;[73] for *V. parahaemolyticus*, the usual infectious dose by food consumption or cross contaminated food ranges between 10^5 - 10^7 cells.[34,35,74] However, highly pathogenic strains such as the ST36 clonal type required lower infectious doses 10^3 – 10^4 cells to cause disease[20], *i.e*. a much lower dose. To our knowledge, no infective dose for skin contact transmission has yet been suggested.

The genes *tdh* and *trh*, major virulence factors typically associated with clinical isolates of *V. parahaemolyticus*[21], are therefore considered strong indicators of potential risk to the public health when detected in environmental isolates. In accordance with other studies[14,53,71,75,76], the percentages of these enteropathogenic *V. parahaemolyticus* detected in the northwest coast of Portugal ranged from 3 – 18% of the total *V. parahaemolyticus*, with higher prevalence in the warmer months. Additionally, *Vibrio* spp. genomes are constantly undergoing evolution through

recombination and horizontal gene transfer, which offers these bacteria a high plasticity and adaptability advantage[9,10], and can explain the high diversity found in the studied isolates.

In this study all *V. parahaemolyticus* isolates harboring virulence genes encoding toxins exhibited multiple antibiotic resistance (AMR). In line with our data, a worldwide meta-analysis studying 2,164 *V. parahaemolyticus* isolates reported high levels of resistance to penicillin.[77] Moreover, by reviewing several areas around the globe antibiotic resistance, such as US, Italy, Brazil, China, India, Malaysia and the Persian Gulf, Elmahdi *et al.*, 2016[78] suggests the exclusion of ampicillin as a treatment for *V. parahaemolyticus* vibriosis. Also, environmental isolates from the Adriatic Sea showed increased resistance to tetracycline[79], which is one of the recommended antibiotics for severe wound infections and bacteremia treatment.[74] More recently suggested antibiotics used for non-*cholerae Vibrio* infections are third generation cephalosporins, fluoroquinolones and doxycycline. [10,74,78] Notwithstanding, while none of the isolates from this study exhibited resistance to doxycycline, intermediate to full resistance to second and third generation cephalosporins such as Ceftriaxone and Cefotaxime, and Ciprofloxacin could be observed, raising concern about the limited therapeutic options available in the future. *V. parahaemolyticus*, a bacterium with a high genome plasticity[80], appears to be developing increased AMR worldwide, consequence of climate change.[81] Indeed, higher temperatures have been proposed to promote by increasing horizontal gene transfer of mobile genetic elements encoding both AMR and virulence-associated genes.[81] Although severe *V. parahaemolyticus* infections are primarily a risk for individuals with underlying health conditions or compromised immune systems, antimicrobial therapy may still be needed for gastroenteritis and wound infections treatment. The emergence of antibiotic-resistant *V. parahaemolyticus* strains reduces treatment options and may pose a significant public health challenge.[81]

A limitation of the present study is the lack of comparison between environmental and clinical samples. These additional data could provide a better understanding of the pathogenic potential of the environmental strains in their natural settings, and consequently the disease burden associated with *Vibrio* exposure through recreational water. Although *V. parahaemolyticus* vibriosis is not a notifiable disease in Europe[26], future studies should address these data gap to enhance the predictive value of environmental monitoring data for public health outcomes.

Conclusions | Conclusões

Recreational waters serve as privileged locations for human exposure to microorganisms with the potential to cause disease, regardless of naturally occurring or anthropogenic source. This study demonstrated that the current Bathing Water Directive, based solely on fecal indicator bacteria is clearly insufficient to assess potential public health risks posed by pathogens, such as *Vibrio* spp.. While during the warmer months of bathing season FIB levels complied with the legal threshold for bathing, *Vibrio* spp. and particularly *V. parahaemolyticus,* revealed the highest abundances. Moreover, *V. parahaemolyticus* harbouring major virulence factors and multidrug resistance were

detected, corroborating the human risk exposure through recreational water. The high diversity of the enteropathogenic *V. parahaemolyticus* isolates found in this study, denote the genetic flexibility and ability to adapt to new ecological settings. The concern is heightened by the fact that the ongoing climate change is expected to support the emergence and spread of those aquatic autochthonous pathogens, especially in northern latitudes. Therefore, rethinking bathing water quality assessment criteria, and design an epidemiological surveillance system under the holistic One Health approach is imperative to ensure the safety of beachgoers.

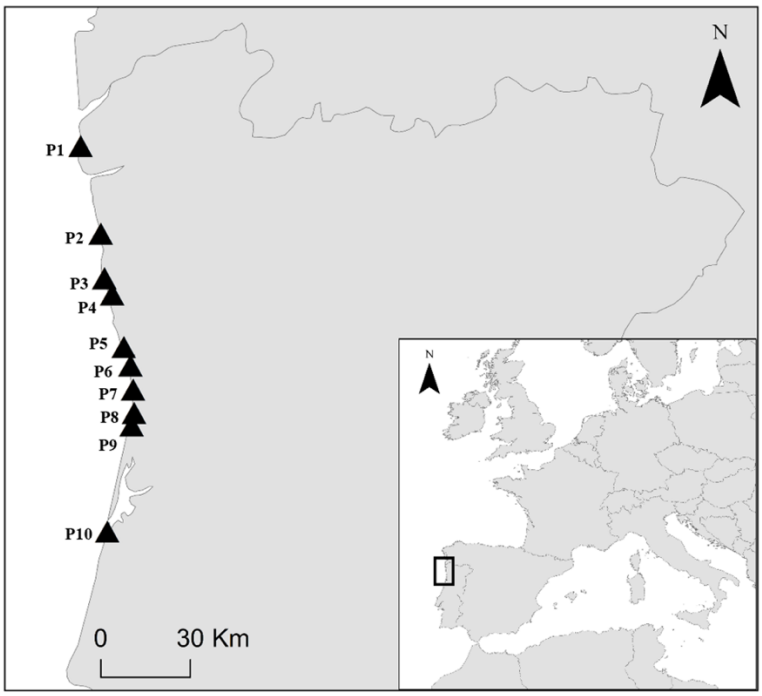
Appendix | Apêndice

Table | Tabela

Table. Primers and conditions used for PCR in this study to detect *Vibrio* spp. and *V. parahaemolyticus* (total, *tdh*+ and *trh*+).

***Vibrio* species**	**Target gene**	**Sequence (5'-3')**	**Amplicon size (bp)**	**Annealing (°C)**	**Extension time (min)**	**Reference**
Vibrio spp.	16S rRNA	567F: GGCGTAAAG CGCATGCAGGT 680R: GAATTCTACCCCCCTCTACAG	120	65	0.5	Thompson *et al*., 2004
V. parahaemolyticus	*toxR*	F-toxRvp: GTCTTCTGACGCAATCGTTG R-toxRvp: ATACGAGTGGTTGCTGTCATG	349	63	1.5	Kim *et al.*, 1999
V. parahaemolyticus	*tlh*	L-tlh: AAAGCGGATTATGCAGAAGCACTG R-tlh: GCTACTTTCTAGCATTTTCTCTGC	450	58	1	Bej *et al.*, 1999
V. parahaemolyticus	*trh*	L-trh: TTGGCTTCGATATTTTCAGTATCT R-trh: CATAACAAACATATGCCCATTTCCG	500	58	1	Bej *et al.*, 1999
V. parahaemolyticus	*tdh*	L-tdh: GTAAAGGTCTCTGACTTTTGGAC R-tdh: TGGAATAGAACCTTCATCTTCACC	269	58	1	Bej *et al.*, 1999

Figures | Figuras



Beach	ID	Latitude	Longitude
Afife	B1	41.78047	-8.87101
Ofir	B2	41.51724	-8.78759
Póvoa Varzim	B3	41.38336	-8.77151
Árvore	B4	41.33426	-8.73977
Matosinhos	B5	41.17604	-8.69252
Salgueiros	B6	41.12033	-8.66514
Aguda	B7	41.04777	-8.65402
Paramos	B8	40.97746	-8.64945
Cortegaça	B9	40.93961	-8.65886
Costa Nova	B10	40.61923	-8.75306

Figure 1. Location of the sampling sites along the NW Portuguese coast. (Made with Natural Earth. Free vector and raster map data @ naturalearthdata.com). Datum WG84.

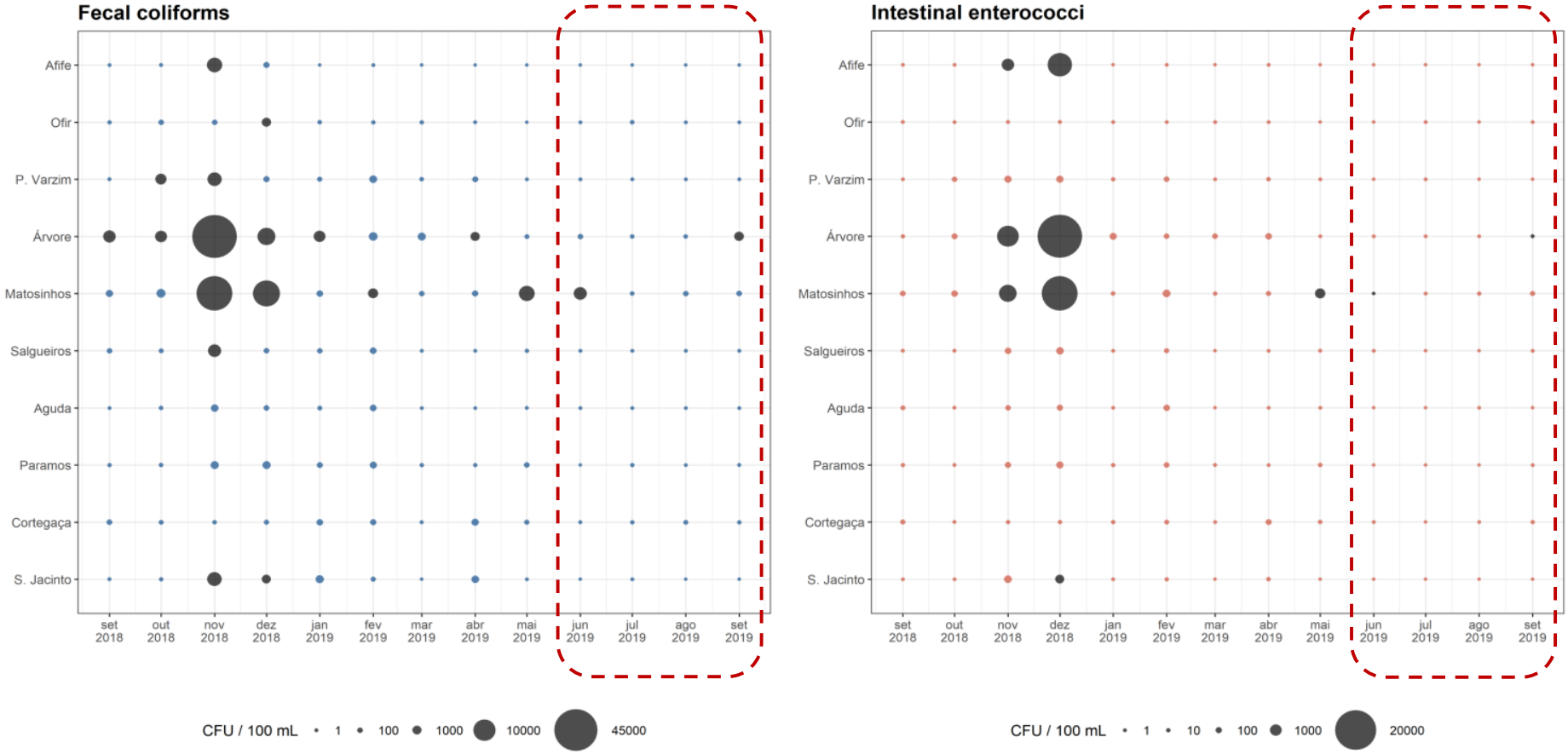


Figure 2. Abundances of fecal indicator bacteria (fecal coliforms on the left, Intestinal enterococci on the right) observed on each recreational water evaluated. The size of the circle represents the colony forming units per 100 mL observed. Black circles represent recreational waters with fecal indicators bacteria above the legal threshold for bathing (1000 CFU / 100 mL for *E. coli* and 300 CFU / 100 mL for EI). Red dashed rectangle limits the official bathing season months.

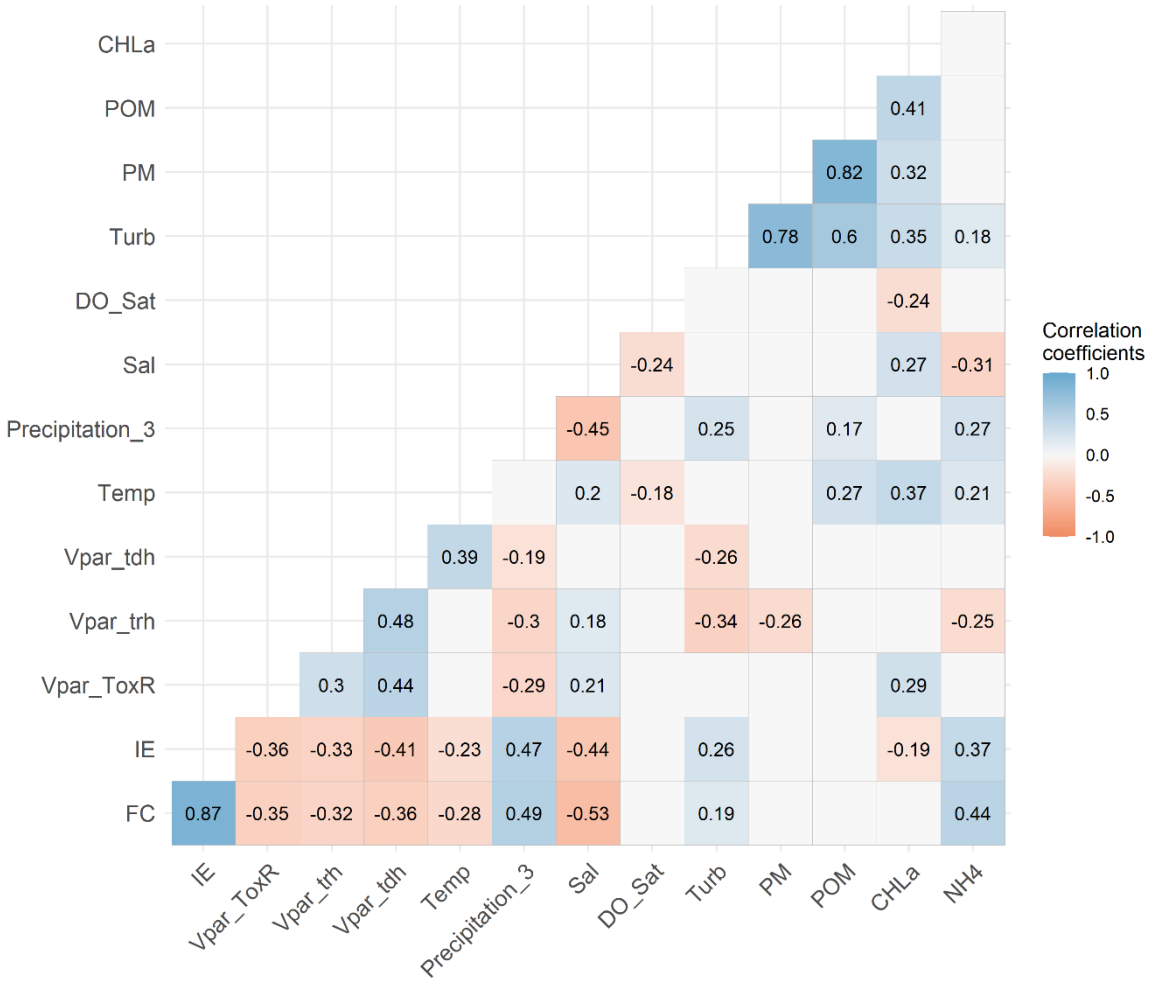


Figure 3 Matrix of Spearman's correlations between environmental variables, fecal indicators and *V. parahaemolyticus* abundances in the recreational waters studied. All correlations shown are significant at $p < 0.05$. FC – fecal coliforms; IE – intestinal enterococci; Vpar_ToxR – total *V. parahaemolyticus*; Vpar_trh – *trh*+ *V. parahaemolyticus*; Vpar_tdh – *tdh*+ *V. parahaemolyticus*; Temp – temperature; Precipitation_3 – precipitation Lag 3 days; Sal – salinity; DO_Sat – oxygen saturation; Turb – turbidity; PM - particulate matter; POM – particulate organic matter; Chl*a* - chlorophyll *a*; NH4 – ammonium.

Figure 4. Abundance of *Vibrio* spp. observed on each recreational water evaluated. The size of the circle represents the MPN / mL observed.

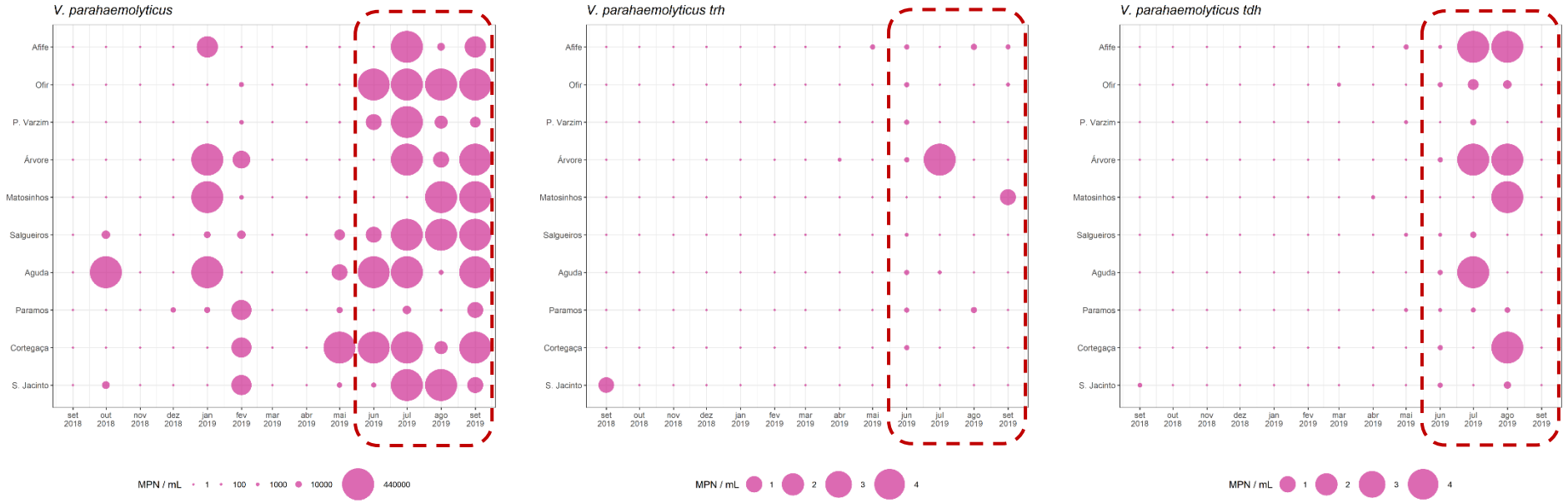


Figure 5. Abundance of total *V. parahaemolyticus* and potentially pathogenic (*trh+* and *tdh+*) observed on each recreational water evaluated. The size of the circle represents the MPN / mL observed. Red dashed rectangle limits the official bathing season months.

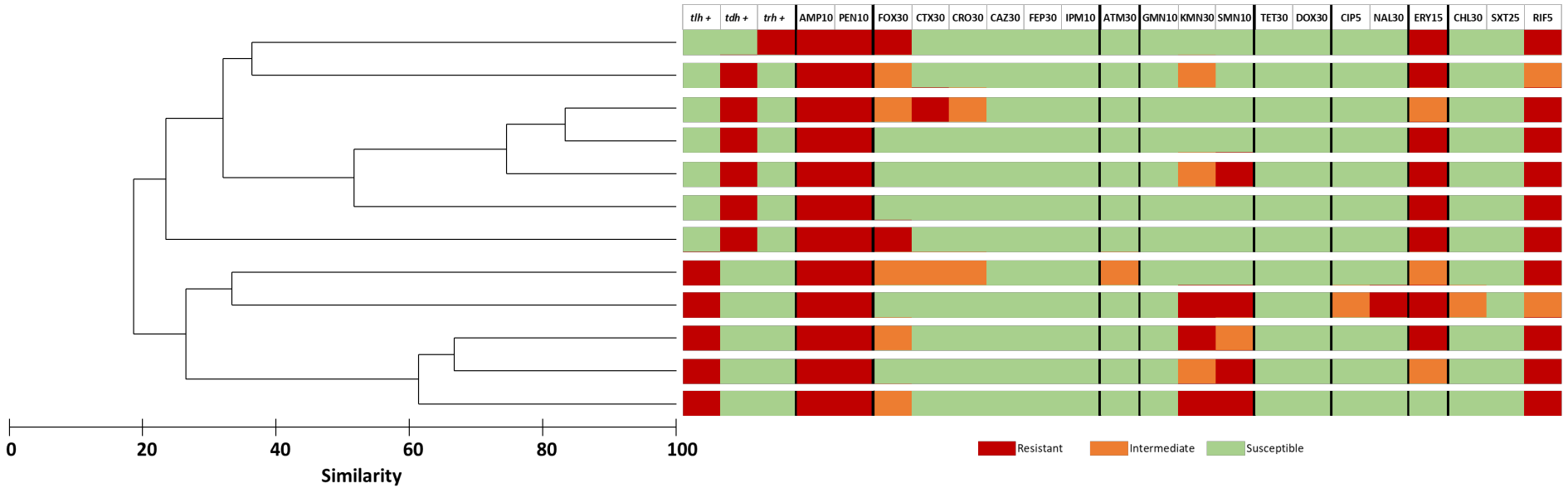


Figure 6. Hierarchical cluster analysis based on ERIC-PCR fingerprints and heat map of the virulence associated genes and antibiotic resistance profiles of the *V. parahaemolyticus* isolates. *tlh*+ - thermolabile hemolysin; *tdh*+ - thermostable direct hemolysin; *trh*+ - thermostable-related direct hemolysin. Thicker vertical lines divided the 9 different antibiotic classes used. Penicillins – Penicillin 10 µg (PEN10), Ampicillin 10 µg (AMP10); Cephalosporins – Cefoxitin 30 µg (FOX30), Cefotaxime 30 µg (CTX30), Ceftriaxone 30 µg (CRO30), Ceftazidime 30 µg (CAZ30), Cefepime 30 µg (FEP30); Carbapenems – Imipenem 10 µg (IMP10); Monobactams – Aztreonam 30 µg (ATM30); Aminoglycosides – Gentamicin 10 µg (GMN10), Kanamycin 30 µg (KNM30), Streptomycin 10 µg (SMN); Tetracyclines - Tetracyclin 30 µg (TET30), Doxyciclin 30 µg (DOX30); Fluoroquinolones – Ciprofloxacin 5 µg (CIP5), Nalidixic acid 30 µg (NAL30); Macrolides – Erythromycin (ERY15), Miscellaneous agents – Chloramphenicol 30 µg (CHL30), Trimethoprim – Sulfamethoxazole 25 µg (SXT25) and Rifampicin 5 µg (RIF5).

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List of publications | Lista de Publicações

At the time of submission of this work, the present data has already been accepted and presented on the *CONGRESS OF MICROBIOLOY AND BIOTECHNOLOGY 2019*, *FEMS 8TH CONGRESS OF MICROBIOLOGISTS 2019, IJUP 13th MEETING OF YOUNG RESEARCHERS OF UNIVERSITY OF PORTO 2020, and WORLD MICROBE FORUM 2021*.

Certificates and abstracts supporting the aforementioned presentations are attached at the end of the thesis.

INSTITUTO DE **CIÊNCIAS BIOMÉDICAS ABEL SALAZAR**

MICRO BIOTEC 19

CONGRESS OF **MICROBIOLOGY** AND **BIOTECHNOLOGY 2019**

December 5th-7th, 2019
University of Coimbra (Pólo II)

BOOK OF ABSTRACTS

SPM
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UNIVERSIDADE D COIMBRA

I1. Environmental Microbiology and Biotechnology

P28. BeachSafe: characterization of *Vibrio parahaemolyticus* isolates in Portuguese bathing water

Tomás Araújo, Adriano A. Bordalo, Ana Machado
ICBAS—UP, Institute of Biomedical Sciences Abel Salazar—University of Porto, Porto, Portugal; CIIMAR—UP, Interdisciplinary Centre of Marine and Environmental Research of the University of Porto, Matosinhos, Portugal

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Vibriosis outbreaks associated with recreational bathing are on the rise in the Northern Hemisphere linked to the ongoing climate changes. Since members of the genus *Vibrio* are gram negative bacteria autochthonous in marine and estuarine environments, its monitoring become essential for public health assurance. In the developed world, *V. parahaemolyticus* is recognized as a major agent causative of illness of non-cholera related *Vibrio* infections, including gastroenteritis and septicemia. Water samples were collected from 10 Atlantic beaches (NW Portugal) over a 15 months' period. Thiosulfate-citrate-bile salts-sucrose agar (TCBS) and Chromoagar *Vibrio* medium were used for presumptive identification and *V. parahaemolyticus* isolation. PCR targeting the *toxR* gene was used to confirm the identification of the *V. parahaemolyticus* isolates, and subsequent PCR targeting genes associated with coding toxin production were assayed (*tlh*, *trh* and *tdh*). Of the 80 confirmed *V. parahaemolyticus* isolates, the *tlh* gene encoding the thermolabile hemolysin was successful found in 16% of the environmental isolates, whereas 11 and 3% exhibited the thermostable direct hemolysin and thermostable-related direct hemolysin, respectively. The assessment of antibiotic susceptibility to 13 antibiotics was carried using the Kirby-Bauer disc diffusion method. The resistance patterns exhibited a wide variability with the majority of the isolates showing resistance at least 3 antibiotics. B- lactam, aminoglycosides and macrocyclic antibiotics revealed the highest number of resistant isolates. Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) and Multiple-Locus Variable-Number Tandem-Repeat Analysis (MLVA), used to identify differences in phylogeny among the pathogenic isolates, displayed higher diversity. The results of this study highlighted the potential public health risk associated with the presence of pathogenic antibiotic resistant *V. parahaemolyticus* in recreational waters. Furthermore, the study revealed the need for monitoring and alert tools development for these natural occurring agents in order to guarantee a BeachSafe to users, especially taking into account the climate change world reality.

This work was funded by the Project BeachSafe (PTDC/SAU-PUB/31291/2017), co-financed by COMPETE 2020, Portugal 2020 and the European Union through the ERDF, and by FCT through national funds.

Certificate

We certify that

Tomás Araújo, Adriano A. Bordalo, Ana Machado

presented the **Poster**

BeachSafe: Characterization of Vibrio parahaemolyticus isolates in Portuguese bathing water

at the Microbiotec'19 Congress, organized as a collaboration between the Portuguese Microbiology Society (SPM) and the Portuguese Biotechnology Society (SPBT), held at the University of Coimbra, Pólo II, from 5 to 7 December, 2019.

7th December 2019

Paula Vasconcelos Morais – Co-Chair
(CEMMPRE, UC)

Jorge Rocha – Co-Chair
(CIEPQPF, UC)

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ABSTRACT BOOK

FEMS 2019

8th Congress of European Microbiologists

In collaboration with sfam society for applied microbiology

7-11 July 2019 | Glasgow, Scotland | www.fems2019.org

PW127 Beachsafe: vibrio dynamics in bathing water and associated human health risk

Ana Machado[1;2], Eva Amorim[1], Tomás Araújo[1], Adriano A. Bordalo[1;2]

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Background: Vibriosis outbreaks associated with recreational bathing are on the rise in Northern latitudes. *Vibrio cholerae*, *Vibrio parahaemolyticus,* and *Vibrio vulnificus* are human pathogens known to cause diarrheal illnesses and wound infections. Ongoing climate changes are expected to further drive their emergence in coastal waters, and the spread of waterborne infectious diseases globally.

Objectives: The aim was to investigate the prevalence of vibrios in bathing waters, understand its dynamics in relation with environmental constrains, and therefore predict potential risk to human health.

Methods: Distribution of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* were investigated in ten beaches over a year, by means of the most probable number-polymerase chain reaction (MPN-PCR). Also, characterization of vibrio isolates, with special attention to genotypes associated with toxicity and antibiotic resistance, was performed.

Results: *Vibrio* sp. were successfully detected in all samples, with abundances ranging 2.23-8.64 Log MPN/L. Although no genes coding toxins were detected, all the potential pathogenic vibrios were found. *V. cholerae* was ubiquous with the highest abundances, whereas *V. parahaemolyticus* and *V. vulnificus* showed lower numbers, peaking in early fall. Temperature, salinity, and particulate matter appear to have a control role. The antibiotic resistance patterns exhibited a wide variability, with isolates from the same *Vibrio* species showing distinct profiles. The results highlighted the potential public health risk associated with pathogenic and antibiotic resistant *Vibrio* species in bathing waters considered safe by the European Union legal criteria.

This work was funded by FEDER (POCI-01-0145-FEDER-031291), and by National Funds from FCT, Portugal.

BOOK OF ABSTRACTS

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- ## 16965 | BeachSafe: quantification and characterization of Vibrio parahaemolyticus in Portuguese bathing waters

Araújo, Tomás D., ICBAS, Portugal
Bordalo, Adriano A., ICBAS, Portugal
Machado, Ana, ICBAS, Portugal

Vibrio parahaemolyticus, a recognized human pathogen responsible for non-cholera Vibrio infections - gastroenteritis, wound infections, and septicemia, are autochthonous to coastal waters. Vibriosis outbreaks in bathing areas are on the rise in the Northern Hemisphere, under the ongoing climate change. The monitoring of these emergent microorganisms is not yet mandatory. Monthly water samples were collected from 10 Atlantic beaches (NW Portugal) over a year. Quantification of *V. parahaemolyticus* was performed by the most probable number-polymerase chain reaction (MPN-PCR). Thiosulfate-citrate-bile salts-sucrose agar (TCBS) and Chromoagar Vibrio medium were used for the presumptive identification of *V. parahaemolyticus*, and confirmation was made by PCR targeting the toxR gene. *V. parahaemolyticus* was always present, with abundances up to 5.64 Log MPN/mL. The higher expression of toxigenic genes occurred during the bathing season. Moreover, those tlh, tdh and trh genes were present in 16, 11, and 6% of the 80 confirmed isolates, respectively. Further identification of differences on the phylogeny among pathogenic isolates was performed by the Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) approach, revealing high diversity. Moreover, antibiotic susceptibility to 13 antibiotics was also assessed, being most of the isolates resistant to at least 3 antibiotics. B-lactam, aminoglycosides, and macrocyclic antibiotics revealed the highest number of resistant isolates. The results of this study highlighted the unaccounted public health risk for beachgoers, and the dire need for new monitoring and alert tools, in order to guarantee a BeachSafe to users, considering the present climate change process.

This work was funded by the Project BeachSafe (PTDC/SAU-PUB/31291/2017), co-financed by COMPETE 2020, Portugal 2020 and the European Union through the ERDF, and by FCT through national funds.

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Certifica-se que Tomás Dias Araújo participou no IJUP'20 – 13º *Encontro de Jovens Investigadores da Universidade do Porto*, que decorreu nos dias 12, 13 e 14 de fevereiro de 2020, na Reitoria da Universidade do Porto, tendo apresentado a comunicação oral com o título "BeachSafe: quantification and characterization of Vibrio parahaemolyticus in Portuguese bathing waters".

Vice-Reitor

Prof. Doutor Pedro Rodrigues

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Bathing Water Quality Assessment And Associated Human Health Risk Under A Climate Change Scenario

Author Block: A. Machado, T. Araújo, E. Amorim, A. A. Bordalo; Inst. of BioMed. Sci. Abel Salazar—Univ. of Porto (ICBAS - UP), Porto, Portugal

Abstract:
Background: There is a growing awareness concerning the emergence and spread of waterborne infectious diseases linked to the ongoing climate changes. Indeed, the number of reported outbreaks of bacterial pathogens linked to recreational bathing are on the rise in the Northern Hemisphere. Ubiquitous in coastal environments, *Aeromonas* sp. and *Vibrio* sp. are recognized as emerging pathogens to humans, known to cause diarrheal illnesses, wound, skin and respiratory infections, and septicemia. However, the current European Bathing Water Directive (2006/7/EC) only takes into account fecal pollution. **Objectives:** The purpose of this study was to investigated fecal indicator bacteria (FIB), together with naturally occurring potential pathogenic bacteria, to determine the robustness of the legal criteria to safeguard the health of beachgoers. **Methods:** Monthly water samples were collected from 10 Atlantic beaches over a 15-month period. FIB were examined by plate count following ISO guidelines, and *Aeromonas* sp. and *Vibrio* sp. abundances were investigated by qPCR. *Vibrio* sp. isolates were characterized regarding antibiotic resistance, virulence factors, and diversity. Multivariate analysis was applied to ascertain the link between environmental signatures and species abundances. **Results:** Overall, during the bathing season FIB met the European legal criteria. *Aeromonas* sp. and *Vibrio* sp. were successfully detected throughout the year, with abundances up to 6 Log bacteria/mL. The temporal seasonality was similar for all species studied (*A. hydrophila*, *A. veronii*, *A. caviae*, *A. media*, *V. cholerae*, *V. parahaemolyticus,* and *V. vulnificus)*, with higher abundances and toxigenic genes expression during the warmer months, coinciding with the bathing season. Temperature, salinity, and particulate matter appear to have a key role in the dynamics of these species. Moreover, the isolates exhibited high genetic diversity and a wide variability of resistance patterns, with resistance at least 3 antibiotics classes (β-lactam, aminoglycosides and macrocyclic). The results highlighted the public health risk associated with the presence of potential pathogenic bacteria in bathing water, that can be considered safe according to the European Union legal criteria. Under a climate change scenario, the development of an integrated microbial risk assessment tool, taking into account natural occurring microorganisms, is pivotal to ensure users safety.
Acknowledgments/ References:
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ASM Sub-track/FEMS Topic (Complete): FEMS - Environmental microbiology and ecology
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