

Effects of temperature and salinity interaction on *Vibrio* spp and *Vibrio parahaemolyticus* in the intercontinental Euro-African Atlantic

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Research Article

Keywords: *Vibrio parahaemolyticus*, pathogen ecology, temperature, salinity, climate change, coastal Europe

Posted Date: June 16th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-607386/v1>

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Abstract

Vibrionaceae include several human pathogens such as *Vibrio cholerae*, *Vibrio vulnificus* or *Vibrio parahaemolyticus*. The risk of vibriosis is increasing worldwide due to the effects of climate change and modified patterns of food consumption, leading to a considerable economic and public health interest in understanding the factors related to the greater abundance of vibrios. Fluctuations in *Vibrio* populations are strongly affected by changes in temperature and salinity, nonetheless, there is substantial variability in their effect and discrepancies in their specific roles. In this study, we analyzed the abundance, and spatiotemporal distribution of *Vibrio* in the Euro-African Atlantic area, focusing the investigation on associations with environmental factors and with an emphasis on *V. parahaemolyticus*. Using membrane-filtration, cultures and MALDI-TOF mass spectrometry, 191 samples from 12 locations were analysed and a biochemical and proteomic profile created. We developed two multivariate linear regression models for the density of *Vibrio* spp (adjusted $R^2 = 0.32$ and 0.27) and a logistic regression model for the occurrence of *V. parahaemolyticus* ($R^2_{\text{Nagelkerke}} = 0.32$, Accuracy = 77%). Including the interaction between sea surface temperature (SST) and salinity in linear regression helps to explain the discrepancies found in several studies on the effect of these variables on the abundance of vibrios.

Introduction

The family *Vibrionaceae* comprises a metabolically and genetically diverse group of heterotrophic bacteria commonly found in all marine and estuarine environments, ranging from coastal to enclosed waters and surface to deep waters [1], [2]. The genera *Aeromonas* and *Plesiomonas* recently separated from the family, leaving 6 genera, including the genus *Vibrio* with 78 species [3], 11 of which are considered to be human pathogens with a varying degree of public health relevance. Of particular interest are *Vibrio cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, which are responsible for cholera, gastroenteritis related to consumption of raw or undercooked seafood, and life-threatening septicaemia following wound exposure to seawater and shellfish consumption, respectively.

Vibrios represent some of the most abundant cultivable bacteria in the ocean [4]. The ecological success of vibrios is reflected by the short multiplication time and association with plants (microalgae, macroalgae, and filamentous cyanobacteria) and animals [5]–[9]. As a consequence, *Vibrio* spp. can rapidly respond to nutrient pulses and become the dominant members of a total bacterial assemblage, resulting in bloom events [10]–[14]. On the other hand, vibrios might be disproportionately subject to predation by protozoa and viruses [15], [16], likely due to their comparatively large size. Thus, *Vibrio* spp. are copiotrophic and opportunistic strategists. They have been found to utilize a wide range of organic carbon compounds, such as chitin, alginate, and agar [8], [17]. These findings suggest that vibrios may exert large impacts on biogeochemical processes in the marine ecosystem [10].

Although *Vibrio* is present throughout the year in temperate countries [18], blooms increase considerably in the warmer months due to the favourable ecological conditions and plankton, and so accumulate in filter-feeding molluscs and other marine animals (Robertson and Tobin, 1983; West, 1989). Numerous studies have investigated the association of *Vibrio* with environmental variables (temperature, salinity, dissolved oxygen, nitrogen, phosphorus, turbidity, pH, chlorophyll a concentrations, dissolved organic carbon (DOC) and zoo- and phyto-plankton taxa) and most concur that temperature and salinity show the highest correlations [5], whereas consideration of additional variables often makes only marginal improvements [19]–[21]. However, a minority of analyses has found temperature and salinity to be non-significant toward explaining *Vibrio* abundance [5], [20], [22]. This inconsistency might be a result of the narrow ranges of data observed for the temperature, such that *Vibrio* abundance varies little, suggesting the magnitude of the correlation may depend on the temperature range examined [5].

There has been a gradual spread to and colonization of new areas linked to the increase in sea surface temperature (SST) as a result of climate change [23]–[27]. According to the European Environment Agency the rise of global sea surface temperature (SST) is one of the major physical impacts of climate change. The effects of climate change include a progressive increase in SST and the melting of the polar ice caps, with consequent changes in salinity, flooding, significant alterations in the distribution of marine species and an increased risk of vibriosis [28]. However, SST in coastal European seas has increased 4–7 times faster over the past few decades than in the global oceans [29]. This local increase in SST has been linked to outbreaks of *Vibrio*-associated human illness caused by *Vibrio cholerae* non O1-non-O139, *V. parahaemolyticus*, and *V. vulnificus* in several European countries [30], and outbreaks occurring during episodes of unusually warm weather [31]. Similar patterns have been observed in vibriosis affecting marine organisms such as fish, bivalves and corals [31]. In Spain, the *Boletín Epidemiológico Nacional* reported 7 outbreaks with 80 cases between 1994 and 2011 [32]. Notably, the first major outbreak in Europe occurred in 1999, with 64 cases associated with the consumption of oysters [33].

Furthermore, an outbreak of 80 cases at a wedding in July 2004, which was linked to the pandemic clone O3:K6 [34], and an outbreak of 100 cases in 2012, after the consumption of crustaceans, possibly contaminated during chilling in ice slurry [35].

Hence, there is considerable public health and economic interest in determining factors correlated to increased abundance of vibrios [5], [36]. Most studies on *Vibrio* distribution have been performed in the Asian and American continent. In a search carried out in March 2021 in Web of Science using the descriptors *Vibrio* (title), salinity (theme) and temperature (theme) without time limitation, 43% of the studies came from America, 31% from Asia and 24 % of Europe. To date, no study has examined the indigenous *Vibrio* populations of the Euro-African Intercontinental waters, an area that includes Spain, Portugal and Morocco. This area is characterized by strong currents and temperature gradients that lead to unique ecological dynamics and includes beaches, fishing ports and aquaculture farms. Furthermore, it is a transit zone of migratory birds between Africa and Europe and merchant ships through the strait of Gibraltar, which overall can lead to the expansion of *Vibrios* between water bodies. Our work aims to determine the presence and abundance of *vibrio* spp (*Vibrio total*), and specifically *V. parahaemolyticus* (Vp), in these waters and associate them with environmental variables.

Materials And Methods

Study area and sample collection.

The study was conducted on the southern coast of Spain between latitudes 36.18571 and 36.59695, and longitudes - 6.30594 and - 5.92076, just before the Strait of Gibraltar separating Europe from Africa where the strong marine currents of the temperate waters of the Atlantic Ocean and the warm ones of the Mediterranean converge. Twelve sampling points were identified along 65 kilometres of coastline, 10 of them located in beach areas, one at the mouth of a seaport (03ca) and one in the area of a fishing port (04ca) (Fig. 1). A total of 191 samples were taken over 16 months, between June 2017 and September 2018, with 85% of the samples being concentrated between June and September, when the frequency of sampling was fortnightly. Samples of beach water were taken 30 cm below the surface in water depth of 1.5 m. At the entrance to the port, they were obtained through a fixed pipe after letting the water run for 5 minutes, and in the fishing port, they were taken 30 cm below the surface next to the ledge of the port. All were taken between 12:00h and 15:00h. Each 500 ml sample obtained was poured into a sterile, non-spill double-sealed plastic container, refrigerated and taken to the University Hospital Puerta del Mar, Cádiz (INIBICA Research Unit). SST was measured in degrees Celsius at the time of sampling using a Checktemp® digital thermometer with probe (range: -50°C to 150°C, resolution: 0.1°C, and accuracy: 20°C ± 0.3°C). Density (in kg/m³) and salinity (in ppt or grams per litre) were measured with a calibrated Atago Refractometer, Master series.

Microbiological methods.

The samples were analysed at the INIBICA Research Unit. For this, 500 ml of water was passed through membrane filters of pore size 10 µm, 2.0 µm and 0.45 µm (Millipore) in series. The filters were inoculated into 2% NaCl alkaline peptone water (pH 8.6) and incubated for 12 hours at 37° C. After incubation, they were seeded on TCBS (Becton Dickinson), blood agar (Becton Dickinson) and ChromID *Vibrio* (BioMerieux), and incubated for 24 hours at 37 °C, following ISO 21872 standards. The isolates obtained were reseeded on ChromID agar and incubated for 24 hours at 37 °C. Afterwards, all isolates were identified by MALDI-TOF MS mass spectrometry (Bruker Daltonics). A direct colony PCR protocol was used for identification, based on the formic acid extraction method and using alpha-Cyano-4-hydroxycinnamic acid (CHCA) as organic matrix. For identification at species level, scores of > 2.2 were considered, using Bruker's Biotyper 3.1 software (Bruker Daltonics). All isolates belonging to the genus *Vibrio* were selected for subsequent cryopreservation to ensure the possibility of conducting further studies, such as determination of the presence/absence of virulence factors by PCR, performing large-scale whole genome sequencing, or detection and phylogenetic analysis of potentially pathogenic strains. The isolates were reseeded in Luria Bertani (LB) agar, 2% NaCl, and incubated for 24 hours at 37 °C. They were then inoculated in 5 ml of LB 2% NaCl broth, incubated for 12 hours at 37 °C, after which 1 ml of each isolate was taken and stored in 50% glycerol at -80 °C. Results are expressed in cfu x 500 ml⁻¹.

Statistical Analysis.

In **univariate analysis**, atypical values were found for *salinity*, *density* and *SST*, and were kept after checking that they did not correspond to errors. Since the Shapiro-Wilk test and histograms showed that none of the variables followed a normal distribution, non-parametric tests were used. Levené's test was used to evaluate homoscedasticity of variables as a prerequisite for certain tests.

In **bivariate analysis**, relationships between quantitative variables were analyzed with Spearman's rank correlation coefficients and scatter diagrams (Supp. Figure 1). The Mann-Whitney U test and Kruskal-Wallis H test were used for associations between quantitative dependent variables and geographical and temporal variables. When significant results were found, pairs were identified with the Wilcoxon signed-rank test, using Bonferroni adjusted p-values. In the case of heteroscedasticity, Welch's test was used, or quantitative variables were categorized as *salinity*. Associations between occurrence of *V. parahaemolyticus* and qualitative variables were analysed by Fisher's exact and Pearson's chi-squared tests, and in cases where the expected frequency was less than 5, by the ANOVA likelihood ratio (LR) chi-squared test. All tests and results can be found in Supp. Table 2.

In **multivariate linear regression** using the response variable *vibrio total*, all explanatory variables were initially included, and those that failed to satisfy the assumptions of collinearity (variance inflation factor, VIF > 2.5), linearity (RESET test, added variable plot), homoscedasticity (Breusch-Pagan test and residuals vs fitted plot) and normality of the residuals (Shapiro-Wilk test and QQ plot) were removed. These checks were performed for each variable added to or eliminated from the model. All variables that met the assumptions of linear regression were left in the final model.

The relationship between the explanatory variables and *V. parahaemolyticus* was analysed using **multivariate binary logistic regression**, following the same procedure as for linear regression. Model adequacy was tested with diagnostic checks for collinearity, linearity, calibration (ROC-pROC-Hosmer-Lemeshow goodness-of-fit test) and discrimination ability (ROC-pROC plot and AUC). Regression coefficient stability was also tested by systematically eliminating predictor variables.

Machine learning techniques were also used to analyse patterns in the data for the purposes of predicting the presence of Vp in seawater. Using *presence Vp* as the response variable, nine supervised classification algorithms were applied: k-nearest neighbours (KNN); naïve bayes (nb); logistic regression (logistic); linear discriminant analysis (LDA); C5.0 decision tree (C50 tree); random forest (rf); stochastic gradient boosting; support vector machines with radial kernel (svmRadial) and neural networks (NNET). Finally, a tenth ensemble model was developed to combine the predictions of the other 9 algorithms and improve the final predictions. The data were previously transformed by scaling and centring, and the absence of zero- or near-zero variance predictors was checked for. To implement the algorithms, the data were partitioned 75%-25% into a training set (training) and a validation set (test) to guarantee the equitable distribution of the variable *Vp*. The model was trained with each algorithm and hyperparameter and validated by repeated k-fold cross-validation (repeated CV) with 10 partitions and 5 repeats involving a total of 50 cross-validation iterations. Once adjusted, the prediction was made on the test set. Second- and third-order polynomials and exponential transformations of environmental variables were tested. The existence of interaction effects between *SST* and *salinity* (*SST*salinity*) was tested. Hypothesis contrasts were established considering a significance level (alpha) of 0.05. R Core Team software (2020), version 3.6.2 was used for statistical analysis.

The spatial analysis was carried out with QGIS software, version 3.4, incorporating a Natural Earth vector map layer (www.naturalearthdata.com), a vector layer with georeferenced sampling points, developed by the research team, and a heat map with filtered images of mean SST in the Atlantic Ocean and Alboran Sea, derived from the NOAA-AVHRR satellite and obtained through the Web Map Service (WMS) of Rediam. The geographic coordinate system used was EPSG:4326 WGS 84.

Results

***Vibrio* spp. abundance (*Vibrio Total*) and prevalence of *V. parahaemolyticus* (*Vp*).**

In the study area, 12 sampling points were identified (Figure 1) where 191 samples were obtained over 16 months, obtaining 371 isolates from different species, 321 (86.5%) of which belonged to 16 species of the genus *Vibrio*. 6 (37.5%) of these species are considered to be human pathogens; they were, in descending order of prevalence: *V. alginolyticus* (88.5%), *V. parahaemolyticus* (26.2%), *V. harveyi* (11.5%), *V. fluvialis* (7.3%), *V. furnissii* (1%), and *V. vulnificus* (0.5%). The first two were isolated at all the sampling points (Supp. Table 1). *Vibrio* spp abundance (*vibrio total*) ranged from 0 to 2.48 log₁₀ with a mean of 1.30 ± 0.55 log₁₀ cfu x 500 ml⁻¹ and *V. parahaemolyticus* ranged from 0 to 2.30 log₁₀ with a mean of 1.34 ± 0.51 log₁₀ cfu x 500 ml⁻¹ (Table 1).

Spatio-temporal distribution.

Vibrio abundance (*Vibrio total*) had significant Spearman correlation with longitude (rho -0.41, p<0.05) and latitude (rho 0.29, p<0.05) (Supp. Figure 1) and was significantly associated with sampling_point (Kruskal Wallis test, p<0.0001). The Wilcoxon test

identified that point 04ca was the only one with differences with other sampling point. Since 04ca corresponds to a fishing port we investigated whether the differences observed there are due to the quality of seawater. To do this, we grouped the water according to their *origin* into three categories (*beach*, *mouth_port* and *fishing_port*) noting that only *fishing_port* is the one that differs significantly from the other two categories, so we regrouped it in a new variable, *sea_quality*, with two categories (*clean* that groups *beach* and *mouth_port* and *no_clean* corresponding to *fishing_port*), noting that they maintain significant differences between the two (Figure 2). A spatial association was also observed for the occurrence of *V. parahaemolyticus* (*Vp*), although, unlike *vibrio total*, *vp* at 04ca was the lowest of all the localizations sampled (Figure 2). Some 98% of samples where *V. parahaemolyticus* was detected were taken at localizations categorized as *clean* seawater (OR 7.17, Pearson's Chi-squared test, $p=0.029$). Nevertheless, this result should be interpreted with caution since the expected frequency in *no_clean* seawater was less than 5. Sampling point was also associated with SST (Supp. Figure 2) showed a significant moderate correlation with latitude ($\rho=0.45$, $p<0.05$) and a weak negative correlation with longitude ($\rho=-0.29$, $p<0.51$) (Supp. Figure 1), confirming that the waters are warmer further to the north west of the study area and closer to the mouth of the Guadalquivir river (Figure 1). There were no significant differences in salinity and density between different sampling points. The minor variation is probably indicative of the fact that estuaries were not included. Both *vibrio total* and *vp* were significantly associated with *year* and *season_year*, but not with *month*, *season* or *julian_day* (Supp. Table 2). In 2018, *vibrio total* was significantly higher than in 2017 (1.4 vs 1.2 \log_{10} ; $p=0.004$) as was *vp* (35.3% in 2018 vs 15.7% in 2017. OR= 2.9, $p=0.003$) (Supp. Figure 3). *Vp* presented a marked heterogeneity in the seasonal series being in any case more prevalent in summer.

Environmental variables.

The effects of SST, salinity and density were analyzed. Density was not significantly associated with *vibrio total* or *vp*. SST varied between 12°C and 28.2°C, with a mean of $21.9 \pm 3^\circ\text{C}$. Salinity varied between 26 and 44 ppt, with 26 being an atypical value, with a mean of 39.5 ± 1 ppt (Table 1). *Vibrio total* was significantly correlated with SST ($\rho=0.29$; $p<0.05$) and indirectly with salinity ($\rho=-0.15$; $p<0.05$) (Supp. Figure 1). *Vp* was not associated with salinity or SST, however, when salinity was segmented into two groups (greater than and less than 39 ppt), a significant association was observed ($\text{OR}_s \leq 39$; $s > 39$: 2.5, 95%CI: 1.25-5.26) (Supp. Table 2). The coefficient of variation (CV: SD/mean) of salinity indicates that its SD represented only 4% of the mean, while that of SST was 14%. The parallel observed between the CV of SST and mean abundance of Vibrios (Figure 3) suggests that the influence of SST on *Vibrio total* may be related more to variations in SST than to mean of SST.

Some authors have hypothesized that the statistical significance of the associations between SST and *Vibrio* abundance depends on the range of the data, since the strength of the correlation with temperature varies according to season (Oberbeckmann et al., 2012; Froelich et al., 2013). In our study, we also observed that the association SST - *Vibrio total* was stronger in spring than in summer ($R^2_{\text{SST}_{\text{spring}}}: 0.18$ vs $R^2_{\text{SST}_{\text{summer}}}: 0.064$ and $\rho_{\text{spring}}=0.40$ vs $\rho_{\text{summer}}=0.24$. $p<0.05$), and that the greatest variation in SST was in spring (CV_{spring}: 10.9% vs CV_{summer}: 9.8%). In autumn and winter, it is not significant due to the scarcity of data.

Adjusted effect of all variables: Multivariate analysis.

Effects on *Vibrio total*. Multivariate linear regression models were developed using the logarithmic transformation $\log(\text{cfu}+0.5)$, *vibrio total*, as the dependent variable, which contributed to compliance with the assumptions of regression. After eliminating variables that did not comply with the assumptions of regression, two significant models were obtained (Table 2). Model 1 included the *longitude*, *sea_quality* and *season_year*. This model satisfied the assumptions of regression analysis and explained 32% of the variability of *Vibrio total*. Model 2, on the other hand, used the SST*salinity interaction instead of *season_year* and obtained a model that explained 27% of the observed variability. It satisfied the assumptions of regression except for the normality of residuals. It should be taken into account however that the residuals of the bivariate regression model with the SST*salinity interaction were normally distributed and the interaction between salinity and temperature is so well known that it justifies its persistence in the model, even despite the limitations for drawing inferences from our results.

The difference between model 1 and 2 therefore is the exclusive selection of *season_year* or the SST*salinity interaction, since the two variables are collinear and the *season_year* coefficient already accounts for the effect of SST and salinity. We chose model 2 since salinity and SST facilitates its interpretation and the interaction helps to understand the combined effect of both variables. The effects are explained in Figure 4. We find that at the same SST, the effect on *Vibrio total* is greater with low salinities and decreases until salinity equal to 42 ppt, from which the effect is reversed (Supp. Figure 4).

Temperature and salinity interact in such a way that each degree of temperature variation modifies (0.54-0.01salinity) times the average *Vibrio total* and for each unit of salinity variation it modifies (0.19-0.01SST) times the average from *Vibrio total*. Therefore, the final effect depends on the interaction of both, and it is not possible to analyze the isolated effects of SST and salinity. This interaction explains why while SST and Salinity were lower in 2018 than in 2017, total vibrio and vp were higher (Figure 5). Polynomial regressions of different orders were tried, but none proved adequate. The standardized coefficients indicate that the variable with greatest influence on the model is sea_ quality, followed by SST, salinity and longitude.

Effects on *V. parahaemolyticus* occurrence (Vp). To explain the presence/absence of culturable *V. parahaemolyticus*, model 3 was developed using multivariate binary logistic regression and the variables *n°_spp*, *origin*, and *s_seg2*, which is the segmentation of *salinity* into two categories (>39 ppt and ≤39 ppt), since the numerical variable was not significant. *SST* and its categories were not ultimately significant but were left in for explanatory purposes. The model explained 32% of the occurrence of Vp in the sampled seawaters ($R^2_{\text{Nagelkerke}} = 0.32$), satisfied the assumptions of logistic regression and showed that the model was well calibrated (Hosmer-Lemeshow test $p = 0.324$) with good discriminatory power (AUC: 0.80, 95%CI: 0.74-0.86) (Table 3).

Figure 6 explains the effects. ROC curve, sensitivity and specificity are shown in Supp.Figure 5.

Supervised machine-learning classification techniques were used to test ten different models based on the nine algorithms described in statistical analysis and one using a stacking ensemble method. Supp.Figure 6 shows the accuracy of training and test results applying the 9 algorithms. Since none of the accuracies fully exceeded the baseline accuracy set (0.738), nor that of multivariate logistic regression model 3 (0.74), we finally chose model 3 as the best explanatory and predictive model of *vp*.

Discussion

The area of this study covers 65 km of the Gulf of Cadiz in the south of Spain and includes open waters (sandy beaches) and closed waters (mouth of the port and fishing port). Because of its strategic position between two continents, it is of great importance for studying the population dynamics of Vibrios. Of the **16 species of genus *Vibrio*** found in water, 6 are human pathogens. The results obtained in the present study revealed an important presence and persistence of *V. alginolyticus* (found at all sampling points, in 53.6% of isolates, and 88.5% prevalence) and *V. parahaemolyticus* (found at all sampling points, in 16.2% of isolates, and mean prevalence of 26.2%, ranging between 5% and 47% depending on sampling point and season). Therefore, these two potentially pathogenic species are both highly prevalent and with wide spatio-temporal heterogeneity. The prevalence of *V. vulnificus* however was low (0.3%) probably due to high average salinity [37]–[39]. We observed that the **number of species** of *Vibrio* isolated is an important marker for evaluating the risk of *V. parahaemolyticus*. For each individual *Vibrio* species found, the risk (odds) of finding *V. parahaemolyticus* is multiplied 2.84-fold (95%CI: 1.9–4.5), which is consistent with the findings of Blackwell and Oliver, 2008, who found that *V. parahaemolyticus* correlated with total *Vibrio* and congeners, as well as coliforms and *E. coli*.

Vibrio total followed a **spatial pattern**, decreasing an average of $-1.1 \log \text{cfu} \times 500 \text{ml}^{-1}$ (95%CI: -1.7, -0.4) for each degree of longitude shift eastward within the coordinates of the study and after adjusting for the remainder of the variables. This implies that there are biotic or abiotic factors other than *salinity*, *SST* and *sea_quality* that vary according to spatial location and affect the abundance of Vibrios, which we believe should be further explored. Studies have demonstrated that abiotic factors such as phosphate, pH, nitrogen, dissolved organic carbon (DOC), turbidity, are not fully suitable to explain variability in *Vibrio total* [5], [19], [20], [40], [41]. Furthermore, even though some studies have found that chlorophyll A is directly associated with *Vibrio total* [20], there are examples showing little to no association between them [21]. Finally, the presence of reservoirs such as copepods or cyanobacteria explains relatively little variance in *Vibrio total* when they are included in a model that incorporates SST [42], [43] or dinoflagellates when salinity is incorporated [41], [42]. Therefore, we think that the spatial pattern that we have identified might be due to the presence of biotic or anthropogenic factors, as these have been demonstrated to influence the population dynamics of Vibrios [44], [45].

Water quality was the most influential factor in the explanatory models. *No_clean* waters had an average of $0.51 \log \text{cfu} \times 500 \text{ml}^{-1}$ (95%CI: 0.29, 0.73) *vibrio total* more than *clean* waters, and was also a very stable result as both linear models had the same regression coefficient. However, the opposite occurred with occurrence of *V. parahaemolyticus*, which was higher in *clean* waters. Relative to samples taken from *fishing_port* waters, there was a 5-fold greater risk of *V. parahaemolyticus* being present if the origin was *mouth_port* (95%CI: 0.35, 142.5) and a 26.06-fold greater risk (95%CI: 3.6, 601.7) if from a beach area, although the scarcity of samples containing *V. parahaemolyticus* in *fishing_port* and *mouth_port* waters gives a high confidence interval for the odds ratio. Although some studies did find a strong association of *V. parahaemolyticus* with *Vibrio total* [40], our discrepancy may not be

surprising. It is known that trends that apply to genus *Vibrio* as a whole do not necessarily apply to those of individual species, and that total vibrios and the potential pathogens, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, correlate with environmental variables that are both shared and different (Takemura et al., 2014). On the other hand, *V. parahaemolyticus* density in fecally-contaminated waters has been observed to correlate more with the levels of zooplankton present than with phytoplankton or fecal contamination as such [46] and could explain the low prevalence found in poorer quality waters. However, further studies are needed to confirm the results, rule out the possibility that *V. parahaemolyticus* is found in a viable but non-culturable state, and investigate ecological and environmental linkages in *no_clean* waters.

Our multivariate regression showed that the **temporal variables** had no effect on the *Vibrio total* or the prevalence of *V. parahaemolyticus*, despite the fact that significant differences were observed in the bivariate analysis between 2017 and 2018 or in the seasonal series. This is because the temporal variations in temperature and salinity were confusing the effect of the *year* and *season_year* variables. This confounding bias would produce collinearity in the regression as the temporal and environmental variables are measuring the same effect and justifies the choice of linear regression model 2 that substituted *season_year* for *SST* and *salinity*.

Numerous studies have established that the **salinity/temperature** binomial is important for the dynamics of *Vibrio* spp [18], [20], [54], [21], [47]–[53] and they are known to be the strongest abiotic correlates among those investigated [5]. In our study area, salinity is high, corresponding to metahaline waters with a median of 39.5 ppt, and very little variation (CV: 0.039), because estuaries were not included. We confirmed that the prevalence of *V. parahaemolyticus* is higher in lower salinity (< 39 ppt), which coincides with other studies [47], [48], and that the risk of finding *V. parahaemolyticus* is 2.76 times higher in salinity \leq 39 ppt (OR 2.76, 95%CI: 1.29–6.12), after adjusting for other variables. The greater prevalence of *V. parahaemolyticus* in lower salinities can be of critical importance for oyster farming in the natural intertidal lagoons and marshlands (*esteros*) that are abundant in this area, where salinity is normally above 40 ppt due to evaporation and the prevalence of *V. parahaemolyticus* is accordingly expected to be lower. Moreover, a recent study showed that pathogenic *V. parahaemolyticus* (trh+) in bivalves was significantly correlated with water salinity, and the probability of finding them decreased 1.45 times with every salinity unit (ppt) increased [55].

Despite *Vibrio total* and *Vp* being both greater in 2018, we found that *SST* and *salinity* were paradoxically significantly lower that year. This apparent inconsistency could be explained due to the interaction of the two variables. A salinity of 42 ppt acts as a traffic light signal changing the sign of the *SST* effect on *Vibrio total*. At salinities lower than 42 ppt, the lower the salinity, the greater the effect of temperature, while above 42 ppt, the *SST* effect is reversed and inversely proportional. Hence, the final effect on *Vibrio total* depends on the interaction of the two variables and not on the isolated effect of each of them separately, and the final result can be positive or negative depending on the combination of variables. It is not possible to analyze the isolated effects of *SST* and *salinity*. We also observed that the **CV curve for SST** paralleled the one for *vibrio total* at different sampling points. This leads us to think that *Vibrio total* is more related to variations in *SST* than to mean temperature, which calls for more in-depth investigations into the relationship between the magnitude, frequency and duration of variations in *SST* and *salinity*, and the development of *Vibrio* spp blooms.

Conclusions

Overall, here we report, that *Vibrio* spp, including the potential human pathogens, *V. alginolyticus* and *V. parahaemolyticus*, are an abundant and dynamic component of the Gulf of Cadiz, with a marked spatio-temporal heterogeneity. 27% of *Vibrio total* variability is explained by the quality of the water, the interaction between *SST* and *salinity*, and the *longitude*, and 32% of *V. parahaemolyticus* variability is explained by the *n*° spp, salinity and origin of the seawater.

The limitations of the study are due to the use of culture and quantification techniques that only detect viable culturable bacteria, and the very small number of samples with presence of *Vp* in *fishing_port* and *mouth_port*, which increases the level of uncertainty. It is necessary to continue the research, increasing the sample size and frequency, measuring variations in *SST* and *salinity*, adding sampling points in estuaries and new biotic and abiotic variables, and extending the study period to increase its statistical power.

Declarations

ACKNOWLEDGMENTS

The authors would like to thank Alcalá Castilla, M., Azagra Rexarch, P., Baro Sánchez, B., Caro Rodríguez, N., Díaz Conejero, JC, Funes Luque, MV, González Cárabe, M., Hidalgo Escobar, A., Maraver Mora, J., Marquez Estévez, L., Muñoz Bascón, M., Navas Corbacho, J., Pacheco Rodríguez, C., Rodríguez Bernal, P., Romero López, E., and Romero Reyes. M.J. for having collected some of the samples and measurements of environmental parameters. We also thank Martín Morales, for processing some of the samples.

Funding. Nothing

Conflicts of interest. None declared.

Code availability. Not applicable

Author contributions. Conceptualization: *Jesus Ruiz-Cayuso, Manuel Rodríguez-Iglesias, Salvador Almagro-Moreno.* Methodology: *Jesus Ruiz-Cayuso, Manuel Rodríguez-Iglesias, Salvador Almagro-Moreno.* Microbiological analysis: *Teresa Trujillo-Soto, Manuel Rodríguez-Iglesias.* Data analysis: *Jesus Ruiz-Cayuso.* Writing-original draft preparation: *Jesus Ruiz-Cayuso.* Writing-review and editing: *Jesus Ruiz-Cayuso, Manuel Rodríguez-Iglesias, Salvador Almagro-Moreno.*

Ethics statement. None required.

Originality-Significance Statement.

Climate change and the higher consumption of raw fish products has led to an increased risk of vibrio infections. Nonetheless, the associations between the environmental factors that directly affect their abundance remain poorly understood. Although temperature and salinity are the two abiotic factors that most explain the variance in Vibrios, there are still gaps in our knowledge regarding their regression coefficients. Using newly identified endemic Vibrio populations from an inter-continental location in southern Europe, here we develop an explanatory model of Vibrio abundance that includes the SST-salinity interaction to explain the discrepancies found in other studies with the effects of these variables.

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Tables

Sea quality	Origin	Sampling point	<i>V. parahaemolyticus</i>			log. cfu vibrio spp x 500 ml ⁻¹ (SD)	SST (°C)		salinity (ppt)	
			Aus (%)	Pres (%)	log. cfu x 500 ml ⁻¹ (SD)		Mean (SD) [CV%]	Median (IQR)	Mean(SD) [CV%]	Median (IQR)
<i>Clean seawater</i>	<i>Beach</i>	01ps (n:16)	12 (75%)	4 (25%)	1.45 (0.74)	1.40 (0.51)	23,1(2,3) [10%]	23,5 (1,9)	39,5(1,4) [4%]	39,5(1,1)
		02ps (n:14)	9 (64,3%)	5 (35,7%)	1.27 (0.77)	1,08 (0.67)	24(1,4) [6%]	24,1 (1,2)	39,3(1,2) [3%]	39,8(1)
		05pr (n:16)	9 (56,3%)	7 (43,8%)	1.37 (0.23)	1,30 (0.42)	23,9(3,1) [13%]	23,8 (2,7)	40,3(2,3) [3%]	40(2,6)
		06ca (n:16)	14 (87,5%)	2 (12,5%)	1.95 (0.07)	1,52 (0.41)	22(2,3) [10%]	22,3 (2,7)	39,2(1,1) [2%]	39(1)
		07ca (n:15)	11 (73,3%)	4 (26,7%)	1.54 (0.05)	1,39 (0.32)	22,8(1,9) [9%]	23 (1,9)	39,4(1,2) [6%]	39,5(1)
		08sf (n:15)	8 (53,3%)	7 (46,7%)	1.38 (0.55)	1,25 (0.50)	22(2,4) [11%]	22,6 (1,9)	39,4(1) [3%]	40(1,5)
		09ch (n:14)	9 (64,3%)	5 (35,7%)	1.10 (0.58)	1,21 (0.55)	21,9(1,7) [8%]	22,2 (2,1)	39,5(1) [3%]	39,8(1)
		10co (n:16)	9 (56,3%)	7 (43,8%)	1.11 (0.52)	1,03 (0.46)	19,4(2,4) [12%]	19,6 (2,8)	39,5(1,1) [3%]	39,8(1)
		11ve (n:15)	13 (86,7%)	2 (13,3%)	1.27 (0.81)	0,94 (0.61)	19,1(3,1) [16%]	20 (4,8)	39,4(0,9) [3%]	39(1)
		12ba (n:16)	12 (75%)	4 (25%)	1.25 (0.81)	0,94 (0.52)	20,7(2) [10%]	21,1 (2,4)	38,5(3,5) [3%]	39(1)
			<i>Mouth_port</i>	03ca (n:19)	17 (89,5%)	2 (10,5%)	0.19 (0.02)	1,47 (0.49)	22,8(3,8) [17%]	23,8 (4)
<i>No_clean seawater</i>	<i>Fishing_port</i>	04ca (n:19)	18 (94,7%)	1 (5,3%)	2.30 –	1,86 (0.49)	21,6(3,8) [18%]	22,9 (4,9)	39,5(1) [9%]	40(1)
		Total (n:191)	141 (73,8%)	50 (26,2%)	1.34 (0.51)	1.30 (0.55)	21.9 (3) [14%]	22.4 (3.7)	39.4 (1.6) [4%]	39.5 (1)

Table 2. Regression coefficients multivariate linear regression models for outcome variable <i>vibrio total</i>										
	Model 1. <i>vibrio total</i> ~ longitude + sea_quality + season_year				Model 2. <i>vibrio total</i> ~ longitude + sea_quality + sst* salinity					
	Coef	95%CI	p-value	VIF	Coef	95%CI	p-value	VIF		
(Intercept)	-7,6473	-11.3, -4.02	4,8E-05	***						
<i>longitude</i>	-1,4529	-2.04, -0.87	2E-06	***	1,0841	-1,069	-1.7, -0.4	1,21E-03	**	1,214
<i>sea_quality</i>			1,1E-05	***	1,1398			1,11E-05	***	1,096
<i>clean</i>	Reference				Reference					
<i>No_clean</i>	0,5036	0,28, 0.72	1,1E-05	***		0,5119	0.29, 0.73	1,11E-05	***	
<i>season_year</i>			1E-06	***	1,0788					
<i>17 spring</i>	Reference									
<i>17 summer</i>	-0,2287	-0.49, 0.04	0,0911	,						
<i>17autwin</i>	-0,288	-0.72, 0.15	0,1937							
<i>18 spring</i>	-0,2208	-0.52, 0.08	0,1494							
<i>18 summer</i>	0,1635	-0.1, 0.43	0,2278							
<i>18autwin</i>	-0,3828	-0.75, -0.02	0,0401	*						
<i>SST</i>						0,5367	0.11, 0.96	0,013	*	
<i>salinity</i>						0,1948	-0.01, 0.4	0,074	,	
<i>SST: salinity</i>						-0,0124	-0.02, -0.002	0,022	*	
Breusch-Pagan test			0,1277					0,955		
Shapiro-Wilk test			0,06					0,001658		
RESET test			0,1733					0,401		
Model p-value			2,1E-11					2,129E-09		
R2 ajusted			0,3222					0,27		
Note: Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1. <i>Vibrio total</i> is the log cfu+0.5										

Predictor	Coeficientes	SE	p-value	OR	OR 95% CI		VIF
					Lower	Upper	
(Intercept)	-8,01	2,17	2.2e-04***	3,3e-04	3,09E-06	1,7e-02	–
<i>n° spp</i>	1,04	0,22	2,03e-06***	2,84	1,88	4,48	1,2
<i>Salinity_segm.</i>							1,1
<i>s>39</i>	reference						
<i>s<=39</i>	1,01	0,40	0,01*	2,76	1,29	6,12	
<i>origin</i>							1,2
<i>fishing_port</i>	reference						
<i>mouth_port</i>	1,61	1,42	0,26	5,00	0,35	142,48	
<i>beach</i>	3,26	1,22	0,01**	26,06	3,65	601,69	
<i>SST</i>	0,06	0,08	0,41	1,06	0,92	1,24	1,1

Note. The coefficients represent the log odds of "Vp = pres" vs. "Vp = aus"

Model Summary. $p < 0.001$. R^2 de Nagelkerke 0.321.

Prediction: Accuracy 0.77 ; Sensitivity: 0.74 ; Specificity: 0.78, for cut-off =Prevalence Vp= 0.26

Model diagnosis: Calibration: Test Hosmer and Lemeshow (GOF). p -value = 0.139. Discrimination. Area under ROC curve (AUC): 0,81 (95%CI: 0.74-0.88). Collinearity: VIF in the table.

Figures

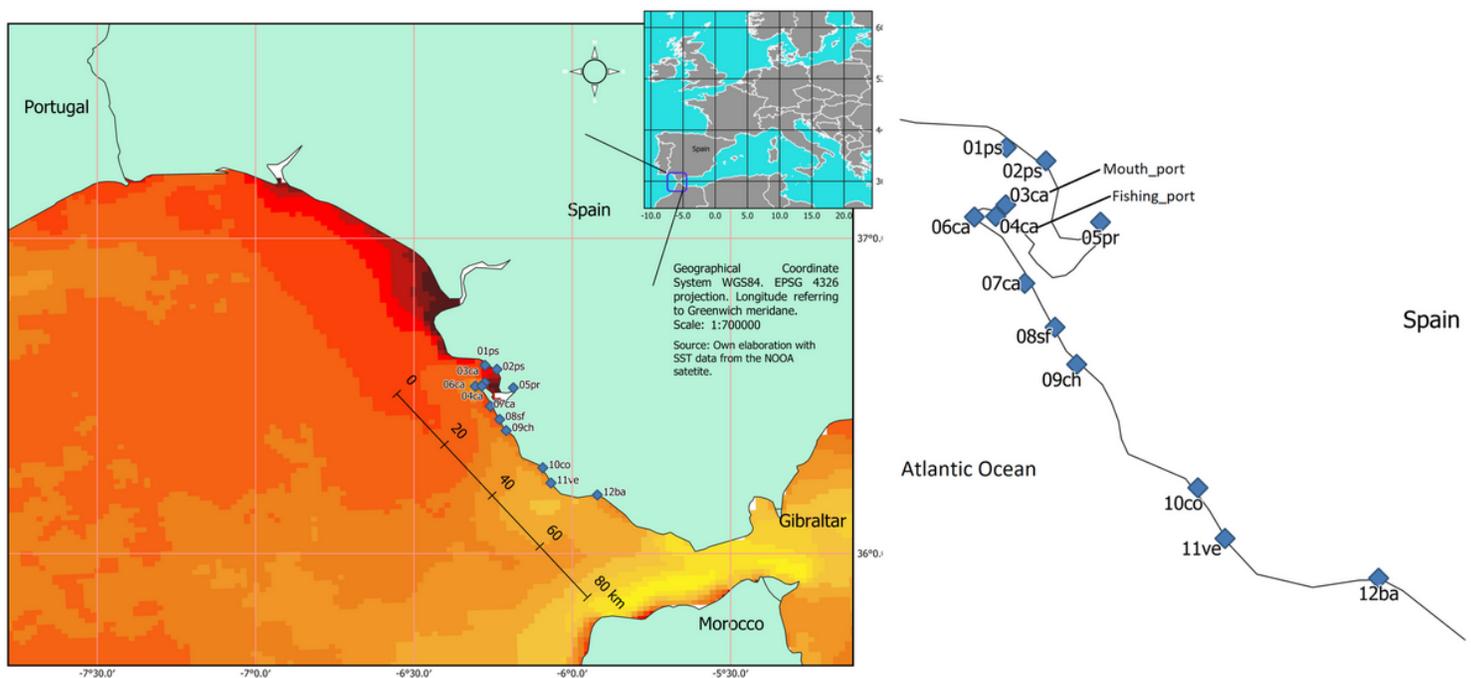


Figure 1

Location of sampling points and Heatmap of Sea Surface Temperature (SST), monthly average leaked August 2018. Satellite images NOAA-AVHRR. Waters are warmer further to the north west of the study area and closer to the mouth of the Guadalquivir river which is

the longest river in south of Spain. Note: ****0, ***0.0001, **0.001, *0.01, .0.05,

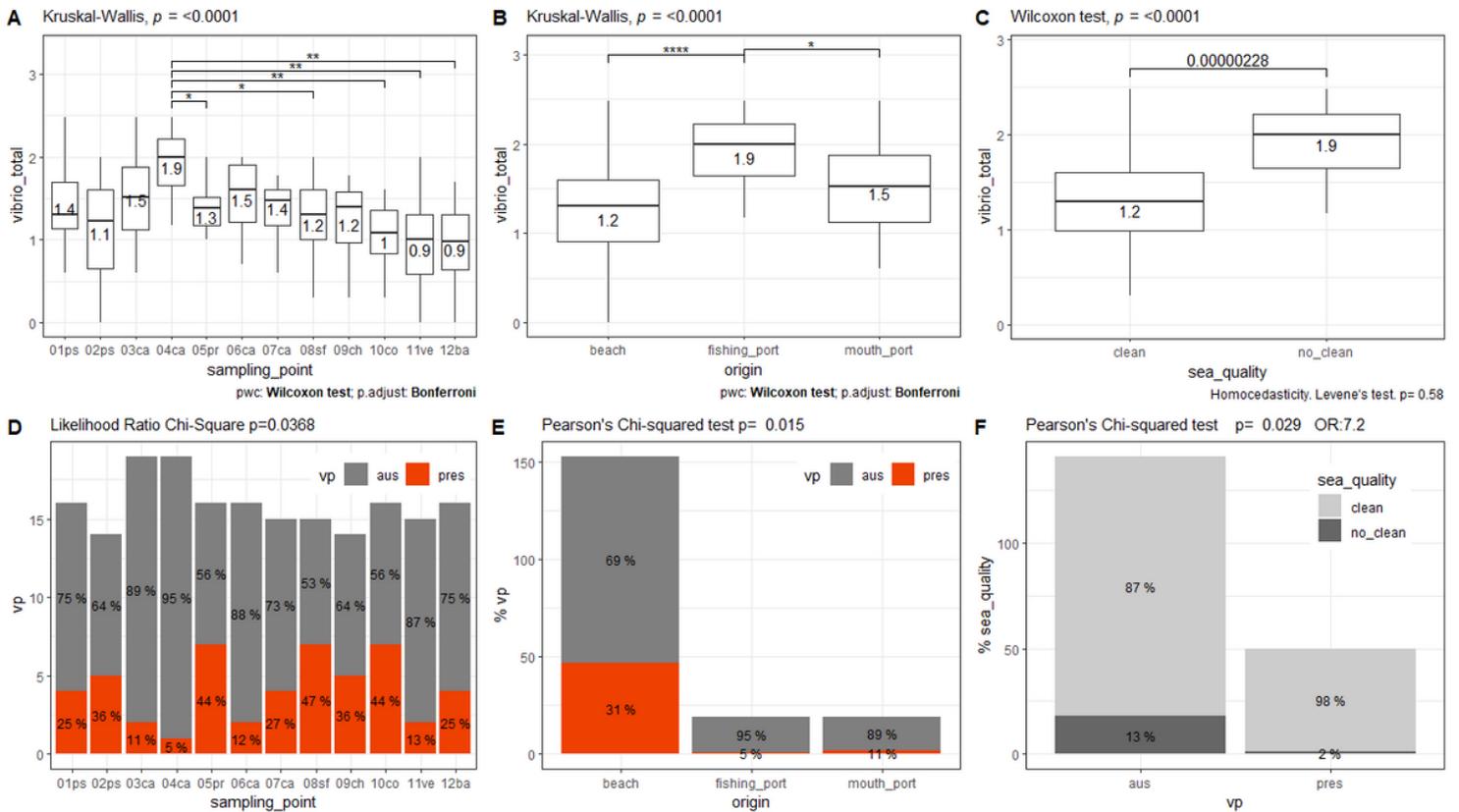


Figure 2

Relationship geographic variables with vibrio total and vp. The seawaters from point 04ca have a significantly higher abundance of Vibrios than the rest of the locations (A). Given that 04ca corresponds to the fishing_port origin and the no_clean seawater quality, these two categories also showed a significant higher abundance of Vibrios than the rest (B, C). The prevalence of Vp in the different sampling points was significantly uneven, ranging between 5 and 47%, with the lowest prevalence being 04ca (D), which corresponds to fishing port (E). About 98% of the samples with the presence of vp were located in clean seawater (OR 7.17) (F).

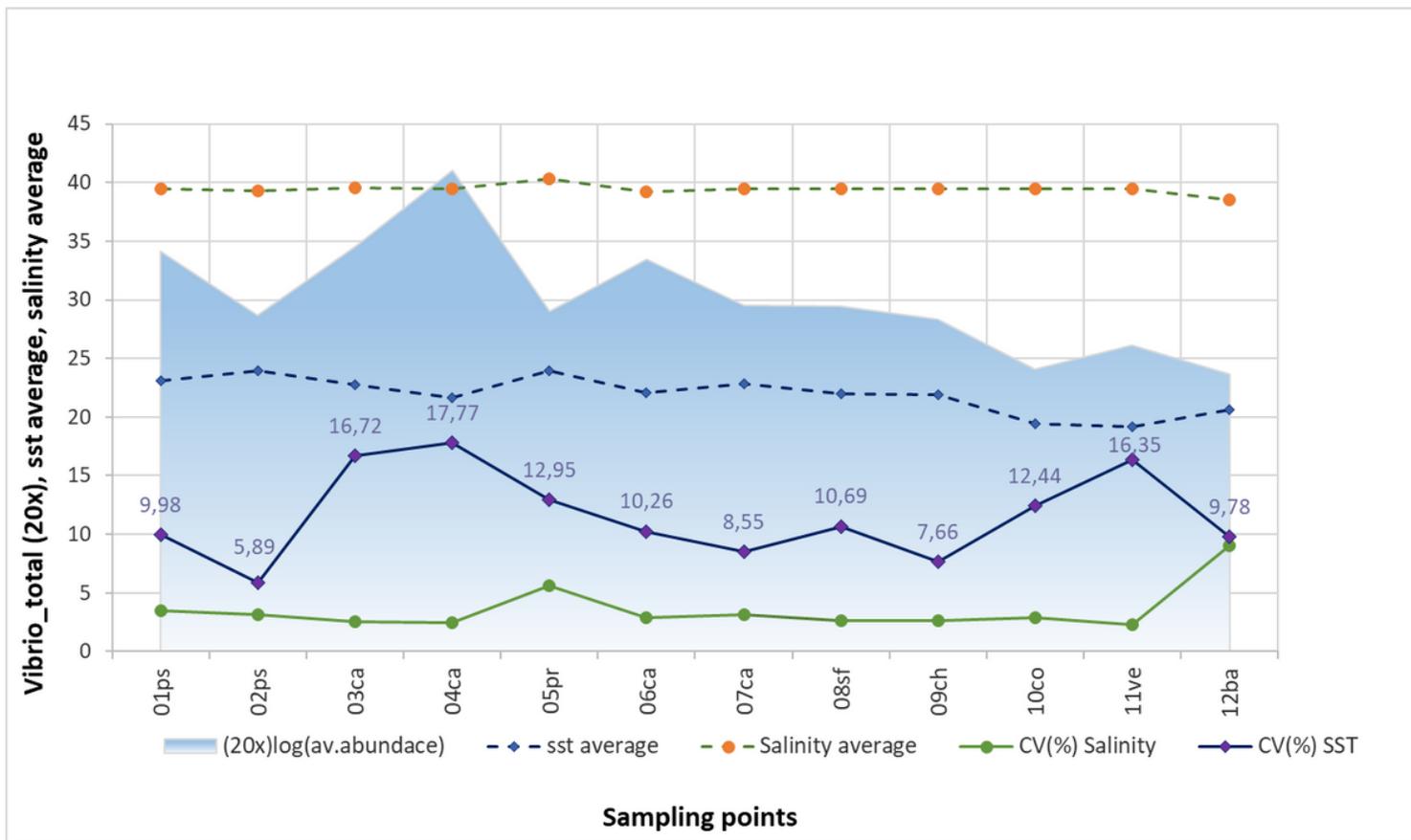


Figure 3

Coefficient of Variation and average of Salinity and SST and 20x vibrio total vs sampling points. The area graph represents the mean density of vibrios spp (log of mean total vibrio, enlarged 20x for better representation). The blue lines represent SST (solid line: CV and dashed line: mean), the green lines represent salinity (solid line: CV and dashed line: mean). The CV of SST presents greater parallelism with the abundance of Vibrios than SST_average that presents little variation.

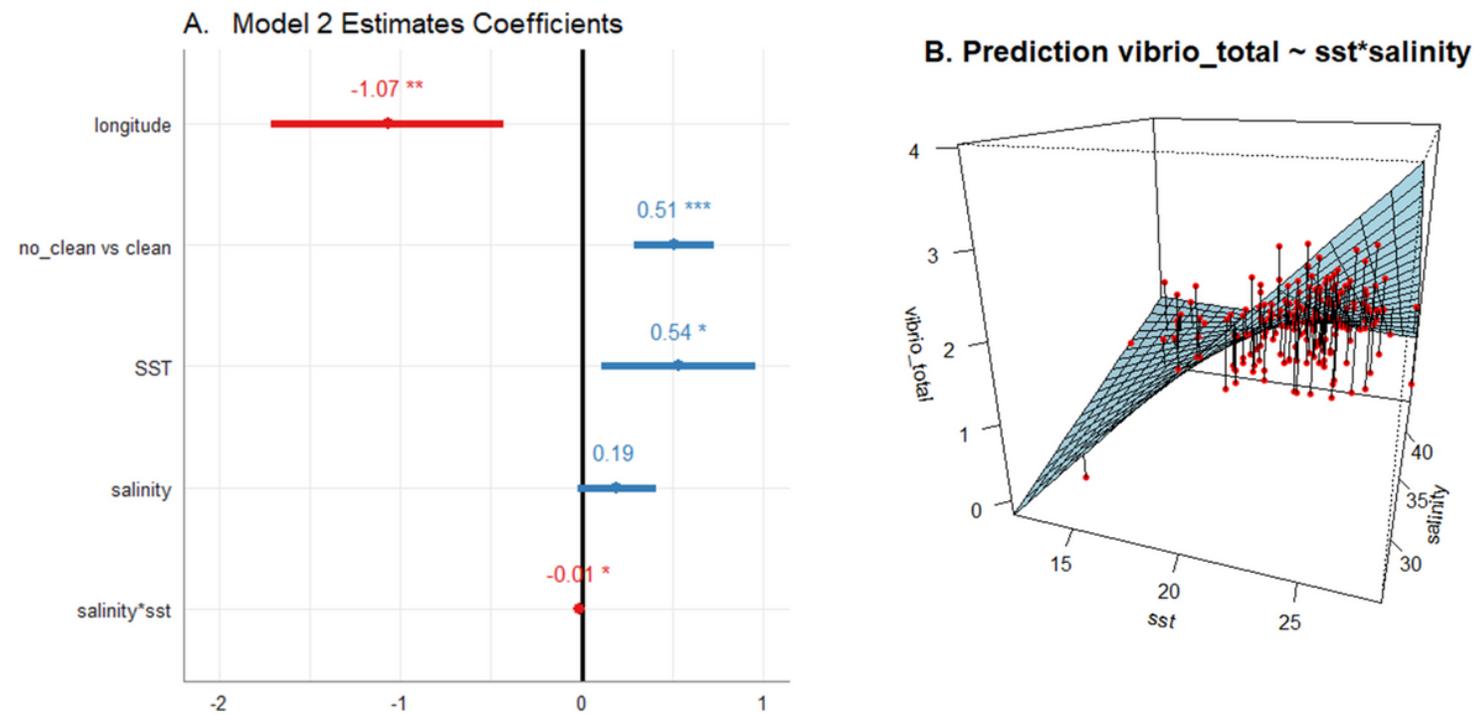


Figure 4

Effects of the SST * salinity interaction on the abundance of vibrios spp as a function of sea quality. A. For each unit of increase in longitude (to the east), there is an average decrease of $-1.07 \log \text{ cfu} \times 500 \text{ ml}^{-1}$ vibrio total (95% CI, $-1.7, -0.4$). Vibrio total in no_clean seawater is an average of 0.51 times greater than clean seawater (95% CI, $0.29, 0.73$). SST and salinity interact producing an average abundance change of $0.54\text{SST} + (0.19-0.01\text{SST}) \text{ Salinity}$. B. The 3D graph on the right represents the effect of the SST*salinity interaction, keeping the other variables constant.

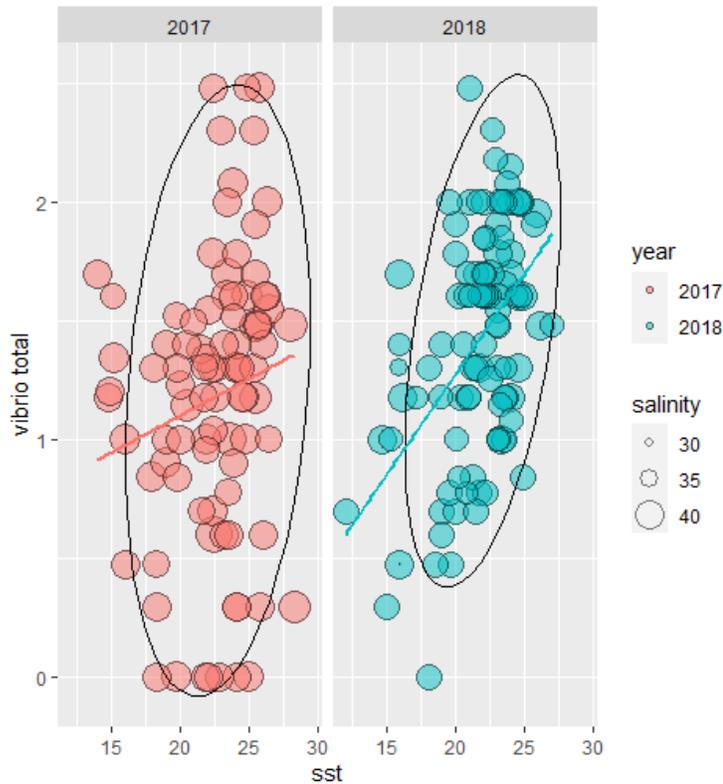


Figure 5

SST and vibrio total scatter plot with salinity bubbles plot. Despite the fact that SST was lower in 2018 (lower relative displacement to the right of the concentration ellipsoid), vibrio total was 0.2 log higher (slope of the regression line), because salinity was lower in 2018 (smaller size of the bubbles) originating a more eugenic combination for the proliferation of Vibrios in 2018.

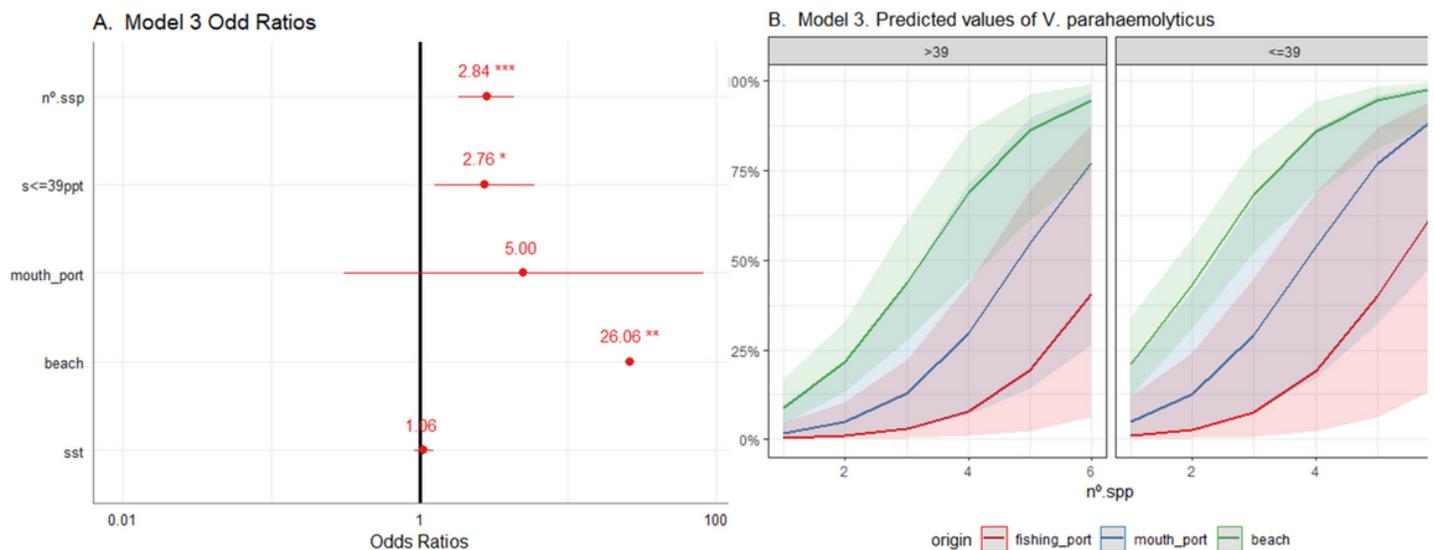


Figure 6

Odd Ratio plot And Predicted values of *V. parahaemolyticus* plot. A. The graph on the left shows the ORs of each variable. SST is not significant, the rest are. The risk of finding Vp is multiplied by 2.84 times (95% CI: 1.9-4.5) for each isolated vibrio species. The presence of Vp at salinities ≤ 39 is 2.76 times (95% CI: 1.29-6.12) greater than at higher salinities. Compared to waters coming from fishing_port, the risk is multiplied by 5 times if it comes from mouth_port (95% CI: 0.35, 142.5) and by 26.06 times (95% CI: 3.6, 601.7) if it comes from beach. The scarcity of observations in mouth and fishing_port gives rise to a wide confidence interval for the dummies, although the origin variable is significant. B. The graph on the right represents the probability of the presence of *V. parahaemolyticus* as a function of the model variables. The maximum probability close to 100% occurs with the detection of 6 species of vibrios, salinity less than or equal to 39 ppt and waters coming from the beach.

Supplementary Files

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- [PublicacionSuplementariorev5.pdf](#)