APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2008, p. 265–274 Vol. 74, No. 1
0099-2240/08/$08.00+0 doi:10.1128/AEM.01307-07

Environmental Determinants of the Occurrence and Distribution of *Vibrio parahaemolyticus* in the Rias of Galicia, Spain▽

Jaime Martinez-Urtaza,[1]* Antonio Lozano-Leon,[1] Jose Varela-Pet,[2] Joaquin Trinanes,[2] Yolanda Pazos,[3] and Oscar Garcia-Martin[1]

Instituto de Acuicultura[1] and Instituto de Investigaciones Tecnológicas,[2] Universidad de Santiago de Compostela, Campus Universitario Sur, 15782 Santiago de Compostela, Spain, and Instituto Tecnolóxico para o Control do Medio Mariño de Galicia, Peirao de Vilaxoán, 36611 Vilagarcia de Arousa, Spain[3]

Received 12 June 2007/Accepted 22 October 2007

Infections associated with *Vibrio parahaemolyticus* on the coast of Galicia (in northwestern Spain) were reported to be linked to large outbreaks of illness during 1999 and 2000. Little information is available about the ecological factors that influence the emergence of *V. parahaemolyticus* infections in this temperate region. We carried out a 3-year study to investigate the occurrence and distribution of *V. parahaemolyticus* at 26 sites located in the four main rias of Galicia in association with environmental and oceanographic variables. *V. parahaemolyticus* was detected in all the areas investigated and throughout the complete period of study with an overall incidence of 12.5%. Salinity was the primary factor governing the temporal and spatial distribution of *V. parahaemolyticus*, whereas seawater temperature had a secondary effect and only modulated the abundance in periods and areas of reduced salinities. Higher occurrence of *V. parahaemolyticus* was observed during periods of lower salinity in autumn, with a total of 61 positive samples (18%) and a mean density of 1,234 most probable number/100 g. *V. parahaemolyticus* was primarily detected in areas of reduced salinity close to freshwater discharge points, where it was found in up to 45% of the samples. Characterization of the isolates obtained from the study resulted in the first identification of two pathogenic *tdh*-positive strains of *V. parahaemolyticus* recovered from the marine environment in Galicia. These isolates showed serotypes identical to and DNA profiles indistinguishable from those of the clinical clone of *V. parahaemolyticus* dominant in infections in Spain in the last 10 years.

Vibrio parahaemolyticus is a natural inhabitant of the marine environments of coastal areas and estuaries worldwide. The presence of the organism has traditionally been confined to warm and temperate geographical areas (11). However, in recent years, the emergence of infections caused by *V. parahaemolyticus* in remote areas of Europe and America (14, 15, 23, 24) has revealed the presence of the organism in regions where it had never previously been reported. The progressive spread of *V. parahaemolyticus* and its colonization of new areas has been related to an unusual increase in seawater temperatures in coastal zones (5, 15, 24). However, little information is available about the environmental variables governing the dynamics of *V. parahaemolyticus* populations in these areas of recent emergence.

V. parahaemolyticus infections were rarely reported in Europe before 1998 (2). The presence of *V. parahaemolyticus* was reduced to sporadic cases reported in different countries without any evidence of epidemiological connection. The epidemiology of the organism in Europe changed significantly when a large number of illnesses associated with *V. parahaemolyticus* were reported in Galicia (in northwestern Spain) in 1999 and 2000. Isolates obtained from the outbreaks in Galicia and from hospitals in other regions of Spain were characteristically *tdh* positive, serotype O4:K11, and belonged to a distinctive clone of *V. parahaemolyticus* not related to other clinical clones that are dominant in other parts of the world (22). Nevertheless, pathogenic isolates of this clone have never been directly recovered from environmental sources or seafood from coastal areas close to the places of emergence of the outbreaks, which makes the environmental origin of the Spanish clinical isolates uncertain.

Despite the importance of the emergence of *V. parahaemolyticus* infections in Galicia, little is known about the presence of the organism in the marine environments of the region. Galicia is one of the most important shellfish-producing regions in Europe, with extensive harvesting of mollusks in the estuarine portions of the coasts, called rias. In the present study, we examined the presence and abundance of *V. parahaemolyticus* in the four main rias in Galicia and evaluated the ecological aspects related to the dynamics of the organism in the coastal areas of this temperate Atlantic region. Additionally, *V. parahaemolyticus* isolates were subjected to intensive analysis to assess the presence of pathogenic specimens in environmental sources.

MATERIALS AND METHODS

Area of study and sample collection. The study was performed in the four most important rias in Galicia, located on the Atlantic west coast of Spain (Fig. 1). The rias of Vigo, Pontevedra, Arousa, and Muros-Noia extend over more than 300 km of coastline and cover an area of 670 km^2. The rias are estuary inlets similar to small fiords and extend from east to west. They were formed by the sinking of river beds, so that each ria joins at least one river at its inland point, which is usually the main source of freshwater. Mollusk cultivation is extensive in the estuarine portions of these rias, with mussel production alone exceeding 200,000

* Corresponding author. Mailing address: Instituto de Acuicultura, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain. Phone: 34 981 528024. Fax: 34 981 547165. E-mail: ucmjmur@usc.es.

▽ Published ahead of print on 2 November 2007.

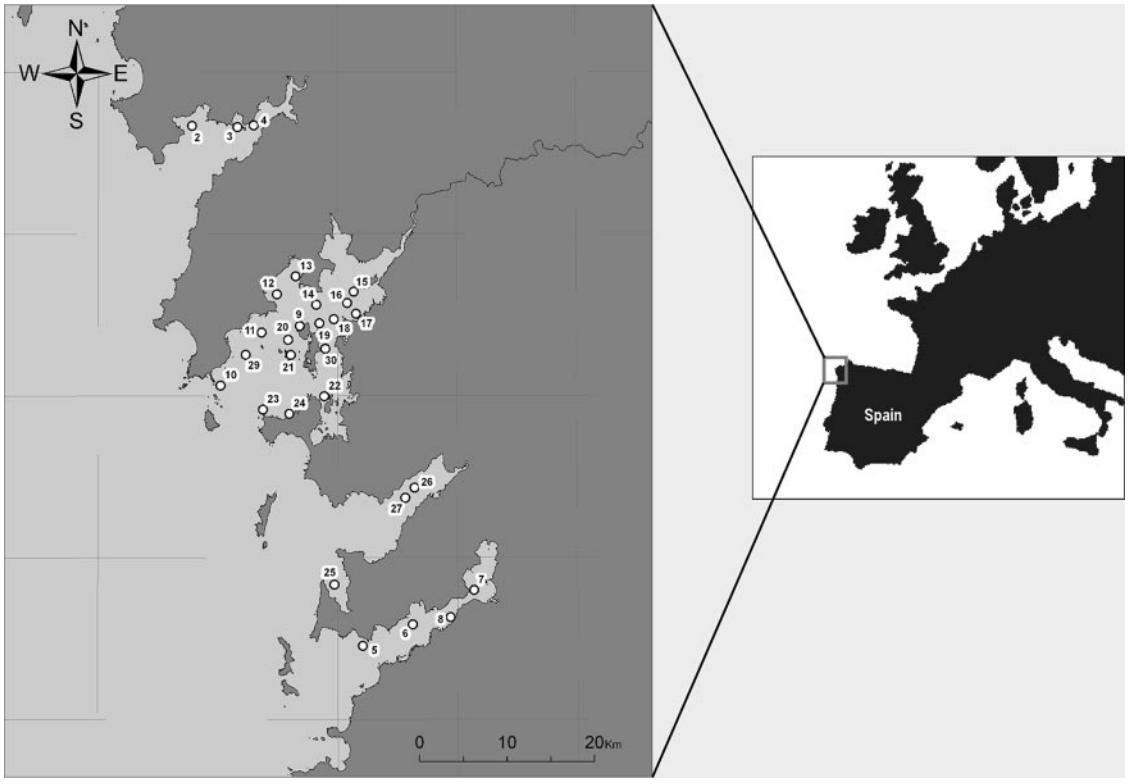

FIG. 1. Area of study and locations of the sampling stations in the coastal areas of Galicia.

tons a year. Mussels are grown on 15-meter-long ropes hung from floating platforms.

A sampling program was designed, including 26 sites located in the four rias, which were distributed according to the size of each ria: 4 in Vigo, 3 in Pontevedra, 16 in Arousa, and 3 in Muros-Noia (Fig. 1). The sampling station at each site comprised one of the ropes of the rafts used to cultivate the mussels. Between January 2002 and December 2004, a total of 1,551 samples of mussels were collected biweekly from the 26 sampling stations.

Bacteriological analyses. One kilogram of mussels was collected at each sampling site. The samples were placed in sterile bags and transported to the laboratory for analysis. In the laboratory, the mussels were immediately removed from the bags and washed in running potable water. Dead mussels or those with broken shells were discarded.

Enumeration of *V. parahaemolyticus* was performed according to the American Public Health Association three-tube most probable number (MPN) procedure (10). Fifty grams of shell liquid and meat was collected in a sterile jar and added to 450 ml of phosphate-buffered saline to make a 1:10 dilution, and the mixture was blended for 120 s with a stomacher. The shellfish homogenate was added to alkaline peptone water in a three-tube MPN dilution series. Due to the low densities of *V. parahaemolyticus* expected in temperate and cold waters like those of Galicia, 10 ml of one 1:10 dilution (containing 1 g of shellfish) and 1 ml of the 1:10 and 1:100 dilutions were selected to inoculate the tubes of three MPN series. The tubes were incubated at 35°C for 24 h. After incubation, a 3-mm loopful from the top 1 cm of each broth tube showing growth was streaked onto two plates of thiosulfate-citrate-bile salt-sucrose (TCBS) agar (Oxoid, Hampshire, United Kingdom). The TCBS plates were incubated at 35°C for 24 h. At least three typical sucrose-negative colonies from each plate were isolated and subjected to identification by biochemical tests on API 20E strips (bioMérieux, Marcy-l'Etoile, France).

Investigation of *tlh*, *tdh*, and *trh* genes. Isolates were confirmed by the presence of the species-specific gene *tlh*. The presence of *tlh* and the virulence genes *tdh* and *trh* were determined by multiplex PCR according to the procedure described by Bej et al. (4). For DNA extraction, the isolate was cultured overnight on a tryptone soy agar plate containing 3% NaCl at 37°C. Several well-grown colonies were chosen and resuspended in 300 μl sterile distilled water and boiled for 15 min to lyse the cells. The lysate was centrifuged, and the supernatant containing DNA was used directly as a template in the PCR. PCRs were carried out in a PTC200 thermocycler (MJ Research, Waltham, MA) with the following reaction conditions: denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94° for 1 min, primer annealing at 58°C for 1 min, and primer extension at 72°C for 1 min. A final extension was performed at 75°C for 5 min. The amplicons were analyzed in a 1.8% agarose gel.

PFGE. Environmental isolates of *V. parahaemolyticus* were compared with human isolates obtained from clinical sources in Spain (22). Pulsed-field gel electrophoresis (PFGE) was performed according to the 1-day (24- to 28-h) standardized laboratory protocol for molecular subtyping of nontyphoidal salmonella by PFGE (7) following a previously described method (22). Chromosomal DNA was digested with 30 U of NotI (Promega, Southampton, United Kingdom) at 37°C for 4 h. DNA macrorestriction fragments were resolved on 1% SeaKem Gold Agarose (Cambrex, Baltimore, MD) in 0.5× Tris-borate-EDTA buffer. DNA from *Salmonella* sp. strain Braenderup H9812 restricted with 50 U of XbaI (Promega, Madison, WI) at 37°C for 2 h was used as a size marker. Pulse times were ramped from 2 to 40 s during an 18-h run at 6.0 V/cm. Restriction patterns were compared by the use of BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium).

Serotyping. Lipopolysaccharide (O) and capsular (K) serotypes of the *tdh*-positive isolates were determined by agglutination tests with specific antisera according to the manufacturer's instructions (Denka-Seiken Ltd., Tokyo, Japan).

Environmental parameters. The environmental parameters included in the study were air temperature, rain, wind, hours of sunshine, rainfall, river flow, upwelling, chlorophyll, phytoplankton counts, seawater temperature, and salinity. The daily ambient temperature was taken as the average of temperatures registered in a day. Wind direction was measured as the time in hours that the wind blew in each of the four prevailing quadrants (northwest, northeast, southwest, and southeast) or no wind (calm). Wind speed was measured as kilometers per day. Rainfall was measured as mm of daily precipitation, and river flow was calculated as the daily average volume of water in m^3/s from the river Ulla. Oceanic parameters (chlorophyll, phytoplankton counts, seawater temperature, and salinity) for each sampling site were obtained from the data provided by the marine environmental-monitoring program that exists in Galicia and is carried out by the Instituto Tecnolóxico para o Control do Medio Mariño de Galicia (Xunta de Galicia Vilagarcia de Arousa, Spain). Daily values of upwelling were

estimated for the point with the coordinates 10°30′W, 42°30′N from the 6-h upwelling values of the Pacific Fisheries Environmental Laboratory (National Oceanic and Atmospheric Administration, Pacific Grove, CA) according to the criteria described by the Pacific Fisheries Environmental Laboratory (http://www.pfeg.noaa.gov/products/las/docs/wind_calc_details.html#interp).

Ambient temperature, rain, wind, hours of sunshine, and rainfall data were provided by the National Weather Institute of the Ministry of Environment and were collected from weather station number 1844 located in the ria of Pontevedra (coordinates, 8°36′59″W, 42°26′24″N; altitude, 107 m). River flow data were obtained from station 544 on the river Ulla (coordinates, 4°20′15″W, 42°50′48″N) and were provided by the Galicia-Coast Network of the Department of Hydraulic Public Domain Management of the Galician Water Department of the Xunta de Galicia.

Spatial analysis. The results of the analyses were processed with the Geographical Information System (GIS) software ArcGIS version 9.1 and the extension Spatial Analyst by ESRI (Redlands, CA). Data were analyzed by the inverse-distance weighted-interpolation algorithm with the following settings: power, 2 (Euclidean distance); 12 points; and variable search radius. The formats of the data were Shapefile (vector data) and GRID (raster data) by ESRI, whereas the vector data source was BCN200 (Base Cartografica Numerica scale, 1:200,000) from the Instituto Geografico Nacional of Spain.

Statistical analysis. The differences in the frequencies of *V. parahaemolyticus* present at different sites and periods were evaluated by chi-square and Fisher's exact tests. Significant differences in the geographical distributions of environmental parameters between rias and inside the rias (north-south and east-west) were evaluated by comparison of pairs of means by analysis of variance and Bonferroni-adjusted multiple *t* tests. When dependent variables were not normally distributed, differences among groups were also assessed by the Kruskall-Wallis analysis of variance of ranks test.

The associations between environmental factors and the presence of *V. parahaemolyticus* were initially analyzed by Pearson correlation coefficients. Environmental conditions that affected the appearance of *V. parahaemolyticus* in the different areas and those related to the increase in *V. parahaemolyticus* counts were investigated separately by the use of independent regression models. Relationships between the presence of *V. parahaemolyticus* and each of the environmental parameters included in the study were initially surveyed by simple logistic regression analysis. Once the significant variables at an individual level were selected, a multiple logistic regression model was conducted with them. In addition, a multiple linear regression was performed with the environmental parameter values and *V. parahaemolyticus* counts to identify the environmental conditions that affected the abundance of *V. parahaemolyticus* in the area. Predicted probabilities and odds ratios (OR) were estimated by logistic regression analysis. The OR was defined as the predicted change in the odds for a unit increase in the corresponding independent variable.

V. parahaemolyticus counts were $\log_{10}$ transformed for statistical purposes, and nondetectable MPN values were replaced by half of the limit of detection. All statistical analyses were carried out with SPSS version 14.0.1 (SPSS Inc.), and the level of significance was set at a P value of <0.05.

RESULTS

Presence and abundance. The presence of *V. parahaemolyticus* was detected at all 26 sites in the four rias and throughout the entire period of study. *V. parahaemolyticus* was detected in 194 of the 1,551 samples investigated (12.5%), and there were no significant statistical differences in the rates of detection in 3 years of study. Significantly higher presence and abundance of *V. parahaemolyticus* was observed in the autumn (Fig. 2), with a total of 61 positive samples (18%) and a mean density of 1,234 MPN/100 g, whereas the highest incidence occurred in November, with 21% of samples positive.

The temperature and salinity of the seawater in the study area ranged from 11.7°C to 20.8°C and 30.9 ppt to 36.2 ppt, respectively. Although the maximum temperatures occurred in summer, the warmest period was characteristically autumn (mean temperature, 15.4°C), and the maximum average temperature occurred in October (16°C). There were no statistical differences in seawater temperatures over the 3 years of the study. Salinity was highest in summer (35.5 ppt), whereas the minimum levels were recorded during the autumn and winter. The highest incidence of *V. parahaemolyticus* characteristically occurred during the phases of decreasing salinity, whereas high levels of the organism mainly occurred when high seawater temperatures overlapped with periods of reduced salinity (Fig. 2).

Although *V. parahaemolyticus* was detected at all the sampling sites studied, its spatial distribution showed a distinctive pattern in each of the four rias investigated (Table 1). A significantly high presence was observed in the rias of Vigo and Muros-Noia, with 26.3% and 18.7% positive samples, respectively, whereas the rias of Arousa and Pontevedra showed significantly lower incidences, with values of 9.8% and 9.2%, respectively. The highest incidences of *V. parahaemolyticus* were detected at sampling stations 3 (20%), 4 (24.2%), 6 (25%), 7 (45%), and 8 (26.3%). Sampling site 7 showed the significantly highest incidence and abundance among all the areas investigated, with *V. parahaemolyticus* present in up to 45% of the samples and a mean density of 3,232 MPN/100 g. All of these sampling sites with maximum occurrence of *V. parahaemolyticus* were in the rias of Vigo and Muros-Noia, and all were located on the northern side of the inner areas of the rias, close to the mouths of the rivers, where the lowest values of salinity were characteristically recorded over the period of the study (Fig. 3). The strong correspondence between the presence of *V. parahaemolyticus* and reduced salinity was also observed in the pattern of distribution of *V. parahaemolyticus* among and within the different rias. The ria of Vigo was the least saline and the coldest of the rias and displayed the highest values of incidence and abundance of *V. parahaemolyticus* (26.3%). By contrast, the ria of Pontevedra was significantly more saline and displayed the highest temperatures, with a significantly lower incidence of *V. parahaemolyticus* (9.2%) than in the other rias. Similarly, the sampling sites with the highest incidence of *V. parahaemolyticus* (sites 4, 6, 7, and 8) displayed significantly lower values of salinity and temperature than the other areas. Station 7, the site of maximum presence of *V. parahaemolyticus*, was also the least saline of all of the coastal areas investigated.

Statistical analysis. Interactions between the environmental and oceanographic variables and the presence of *V. parahaemolyticus* were initially surveyed by correlation analysis. The occurrence of *V. parahaemolyticus* was positively and significantly correlated with northwest and southwest winds, river flow, and seawater temperature but negatively correlated with calms, salinity, chlorophyll, phytoplankton, and upwelling. The highest significant association was observed with salinity (correlation coefficient, 0.16) and, more specifically, with the salinity recorded at deeper levels (15 m), for which the significance value (P) was $<10^{-9}$. The presence of *V. parahaemolyticus* was largely influenced by the decrease in salinity and all other parameters that favored the decrease in salinity. Detection of *V. parahaemolyticus* was therefore associated with river flow, which promoted the influx of freshwater in the rias; the presence of northwest and southwest winds, which were dominant in the rainy periods; and calms, which were linked to stable conditions in the absence of rain. The occurrence of *V. parahaemolyticus* was also negatively affected by all of the factors involved in oceanic primary production, such as upwelling,

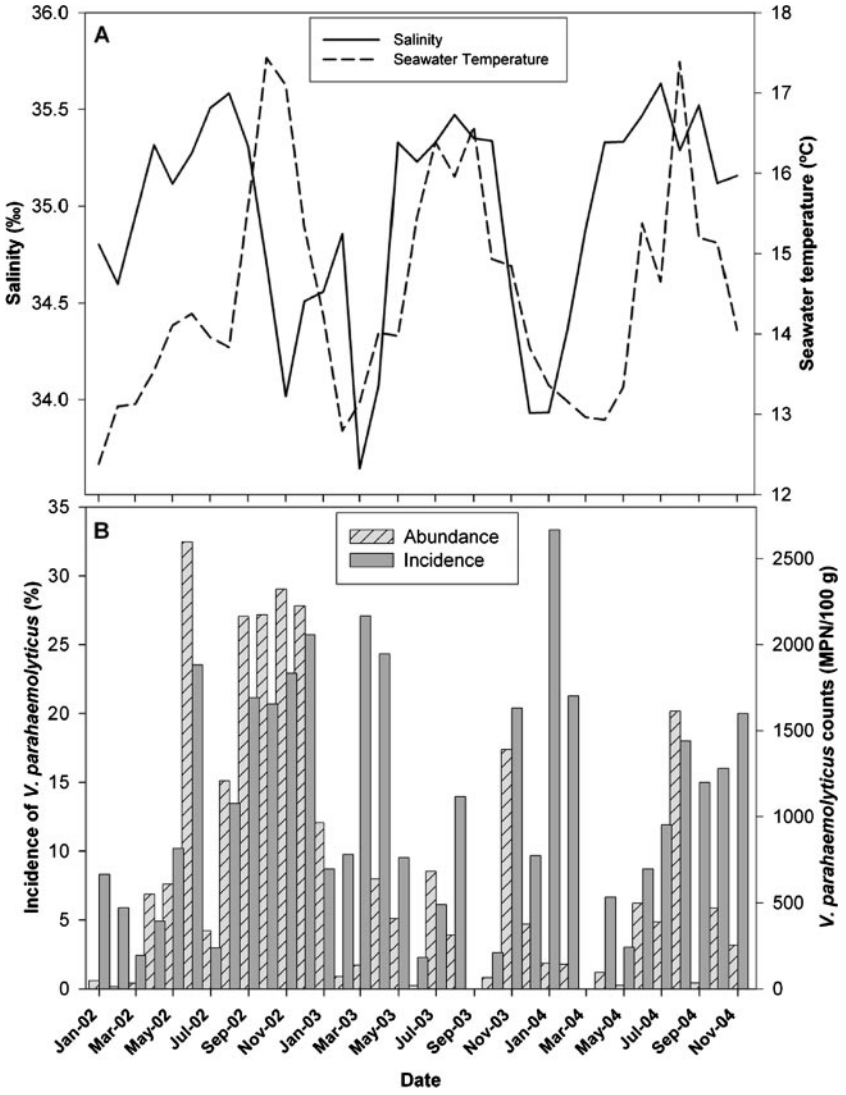

FIG. 2. Variations in salinity and seawater temperature throughout the period of study (A) and distribution of the presence and abundance of *V. parahaemolyticus* during the same period (B).

chlorophyll, and phytoplankton counts, whereas its presence was associated with the concurrence of downwelling periods.

Simple logistic regression analysis of the presence of *V. parahaemolyticus* and environmental variables showed significant relationships ($P < 0.05$) with northwest and southwest winds, salinity at deeper levels, diatoms, and upwelling, with a lag of 1 day. Salinity was the dominant factor affecting the presence of *V. parahaemolyticus* and showed a strong influence on the presence of *V. parahaemolyticus* in the zone. An increment of 1 unit (ppt) in salinity accounted for a reduction of more than 0.5-fold in the probability of detection of *V. parahaemolyticus* ($P < 0.0001$).

To identify those environmental variables that distinctively influenced the abundance of *V. parahaemolyticus*, associations between the explanatory variables and *V. parahaemolyticus* counts were evaluated statistically, first for all of the coastal areas studied and subsequently for the ria of Vigo, where the occurrence of *V. parahaemolyticus* was highest. Apart from the northwest and southwest winds, salinity at deeper levels, and upwelling with a lag of 1 day, the results from multiple linear regression analysis identified seawater temperature as a distinctive significant factor governing the increase in *V. parahaemolyticus* levels ($r = 0.26$; $r^2 = 0.06$). Decrease in salinity was the dominant factor in the model and had a highly significant negative effect on the abundance of *V. parahaemolyticus* ($P < 0.0001$). Results obtained from the analysis of only data from the ria of Vigo offered a better explanatory model ($r = 0.50$; $r^2 = 0.25$) and identified northwest winds, calm periods, salinity, and seawater temperature as the only significant factors that affected the abundance of *V. parahaemolyticus* in the ria. Salinity was the main factor in the model ($P < 0.0001$).

The influences of seawater temperature and salinity on the *V. parahaemolyticus* dynamics in the area were further evaluated by calculation of the probability of detection of *V. parahaemolyticus* in association with variations in salinity and seawater temperature and with both variables simultaneously

TABLE 1. Average incidence and abundance of *V. parahaemolyticus*, salinity, and seawater temperature in the different sampling stations over the period of the study

Ria	Sampling station	No. of samples	No. of positive samples (incidence [%])	Avg abundance (MPN/100 g)	Salinity range (ppt)	Seawater temp range (°C)
Vigo	5	38	3 (7.9)	878	32.4–35.7	12.5–18.5
	6	40	10 (25.0)	1,984	32.2–35.6	11.8–18.2
	7	40	18 (45.0)	3,233	31.3–35.6	11.7–17.8
	8	38	10 (26.3)	1,804	32.2–35.7	11.9–18.2
	Total	156	41 (26.3)	1,991	31.3–35.7	11.7–18.5
Pontevedra	25	63	5 (7.9)	722	30.9–35.9	12.6–18.9
	26	55	7 (12.7)	256	32.8–35.8	12.6–18.2
	27	55	4 (7.3)	94	33.1–35.8	12.5–18.5
	Total	173	16 (9.2)	374	30.9–35.9	12.5–18.9
Arousa	9	68	8 (11.8)	427	33.5–35.7	12.1–20.2
	11	49	2 (4.1)	235	33.3–35.6	12.0–18.1
	12	67	1 (1.5)	10	32.7–35.8	11.9–20.4
	13	61	5 (8.2)	600	32.7–35.8	11.9–20.4
	14	67	7 (10.4)	252	32.4–35.7	11.7–20.8
	15	67	8 (11.9)	415	32.4–35.7	11.7–20.8
	16	67	12 (17.9)	1,202	32.6–35.8	11.7–20.5
	17	67	13 (19.4)	883	31.8–35.8	11.7–20.5
	18	67	3 (4.5)	340	32.9–35.7	11.9–20.5
	19	68	4 (5.9)	334	33.3–35.7	11.9–20.5
	20	68	7 (10.3)	727	33.5–35.7	12.1–20.2
	22	66	7 (10.6)	408	32.1–35.7	12.2–20.0
	23	66	4 (6.1)	219	33.2–35.7	12.3–19.7
	24	67	11 (16.4)	769	33.2–35.7	12.3–19.7
	29	46	2 (4.3)	12	32.9–35.7	12.9–17.8
	30	68	7 (10.3)	725	33.3–35.7	11.9–20.5
	Total	1,029	101 (9.8)	486	31.8–35.8	11.7–20.8
Muros Noia	2	62	7 (11.3)	647	32.9–36.1	12.4–19.8
	3	65	13 (20.0)	964	32.7–36.2	12.5–20.2
	4	66	16 (24.2)	1,500	32.5–35.7	12.4–20.2
	Total	193	36 (18.7)	1,045	32.5–36.2	12.4–20.2
Total		1,551	194 (12.5)	694	30.9–36.2	11.7–20.8

(Fig. 4). The presence of *V. parahaemolyticus* was mainly influenced by salinity (OR = 0.561; $P < 10^{-10}$). A reduction in salinity of 2 units doubled the probability of the presence of *V. parahaemolyticus*. By contrast, seawater temperature showed a weaker effect and contributed to lower predictability of the presence of *V. parahaemolyticus* (OR = 1.169; $P < 0.01$). The combined effects of both of these environmental variables corroborated previous observations about their influence on the temporal and spatial distribution of *V. parahaemolyticus*. The probability of detection increased greatly as values of salinity dropped below 34 ppt. However, seawater temperature alone exhibited a secondary effect in the model and influenced the predicted detection only if the increase in temperature was combined with reduced values of salinity.

Strain characterization. All 2,320 of the presumptive *V. parahaemolyticus* isolates obtained from the study were subjected to biochemical investigation. A total of 464 colonies isolated from the 194 positive samples exhibited the characteristic biochemical profiles of *V. parahaemolyticus* and were confirmed by PCR for the presence of the species-specific gene *tlh*. Most of the remaining 1,856 isolates were tentatively identified as *Vibrio alginolyticus* (1,802 isolates) and *Vibrio vulnificus* (19 isolates). All of the *V. parahaemolyticus* isolates investigated lacked the virulence attributes *tdh* and *trh*, except for two isolates belonging to the same sample, number 25,010, obtained from sampling station 16 located in the interior of the ria of Arousa on 25 November 2003. Both were positive for the *tdh* gene and negative for the *trh* gene and were identified as belonging to serotype O4:K11.

Molecular characterization by PFGE of 23 selected *tdh*-negative environmental isolates and the two *tdh*-positive strains clearly discriminated the isolates according to their genetic traits (Fig. 5). The two *tdh*-positive isolates shared identical restriction patterns and were differentiated from the rest of the environmental isolates. They showed identical serotypes and a restriction pattern that was indistinguishable from that of the clinical strains of *V. parahaemolyticus* isolated in different locations in Spain over the last 10 years and belonging to the Spanish clinical clone. By contrast, all the *tdh*-negative environmental isolates presented high genetic diversity and showed unrelated PFGE profiles. No relationships among isolates from the same ria or among isolates obtained during the same period of study were observed.

DISCUSSION

The results obtained in the present study revealed an important presence and persistence of *V. parahaemolyticus* in

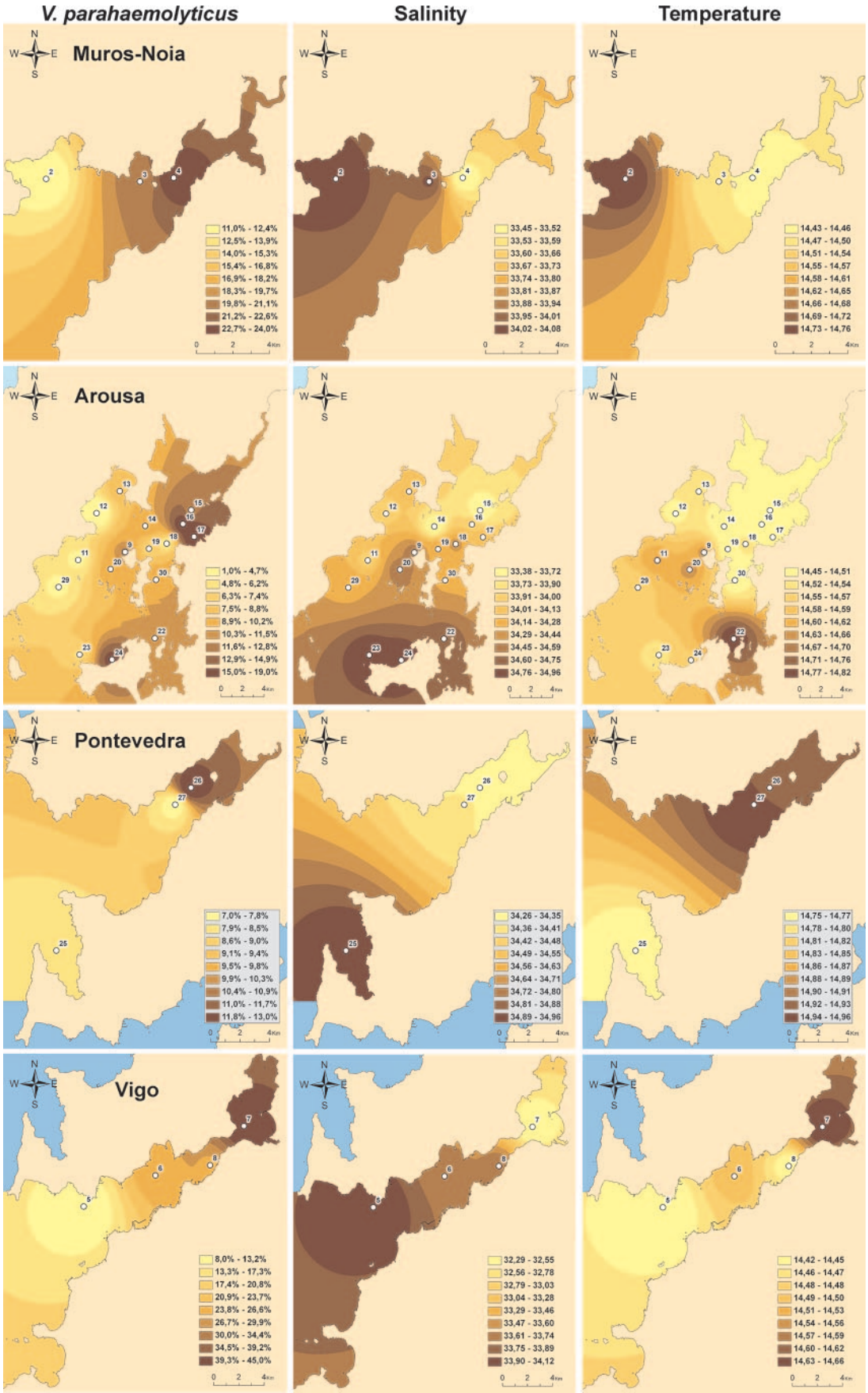

FIG. 3. GIS maps showing the spatial distributions of *V. parahaemolyticus*, salinity, and seawater temperatures in the rias of Muros-Noia, Arousa, Pontevedra, and Vigo. Note the different values associated with the color scales in the maps.

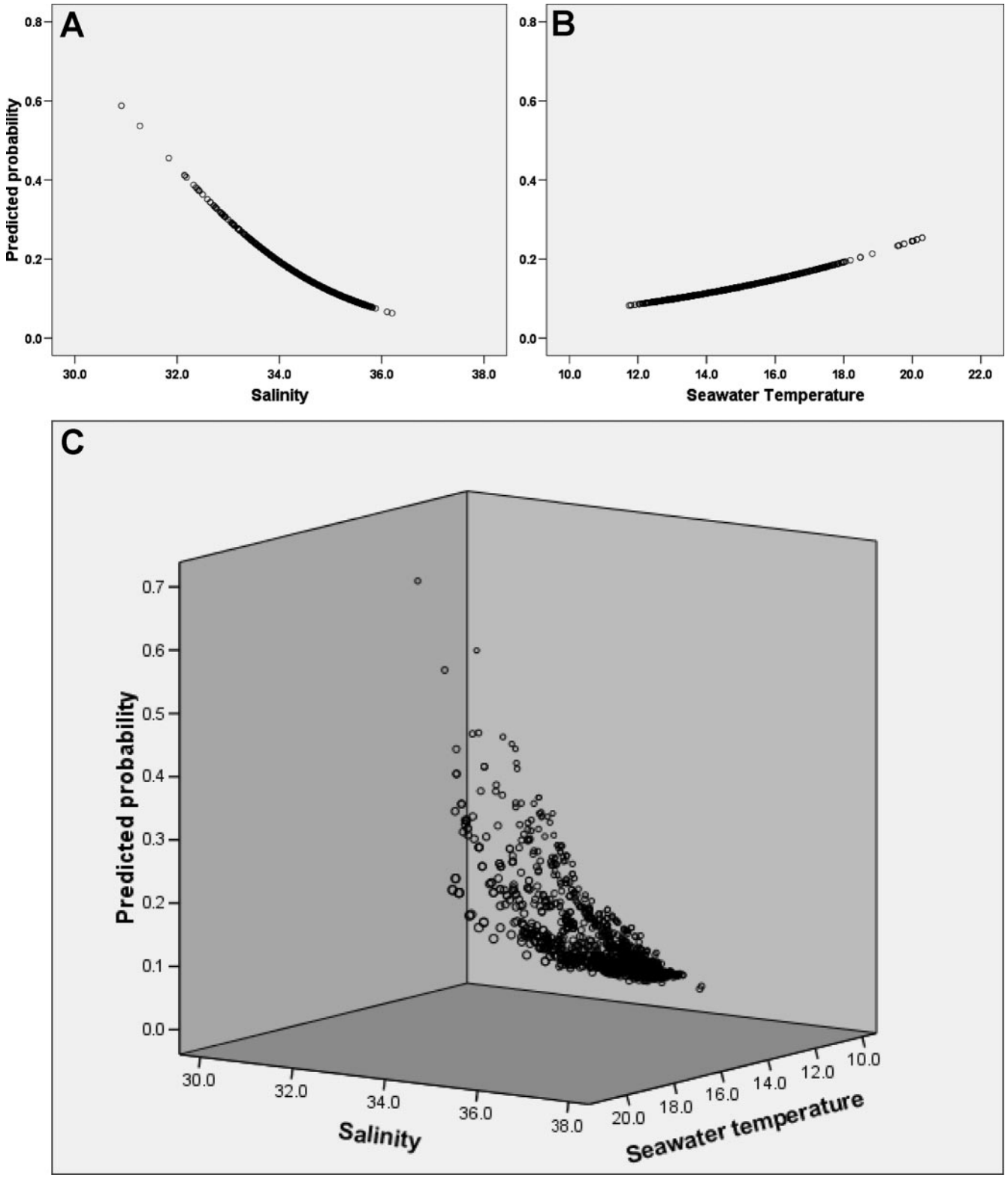

FIG. 4. Predicted probabilities for the detection of *V. parahaemolyticus* in association with the values of salinity (A) and seawater temperature (B) and both variables simultaneously (C).

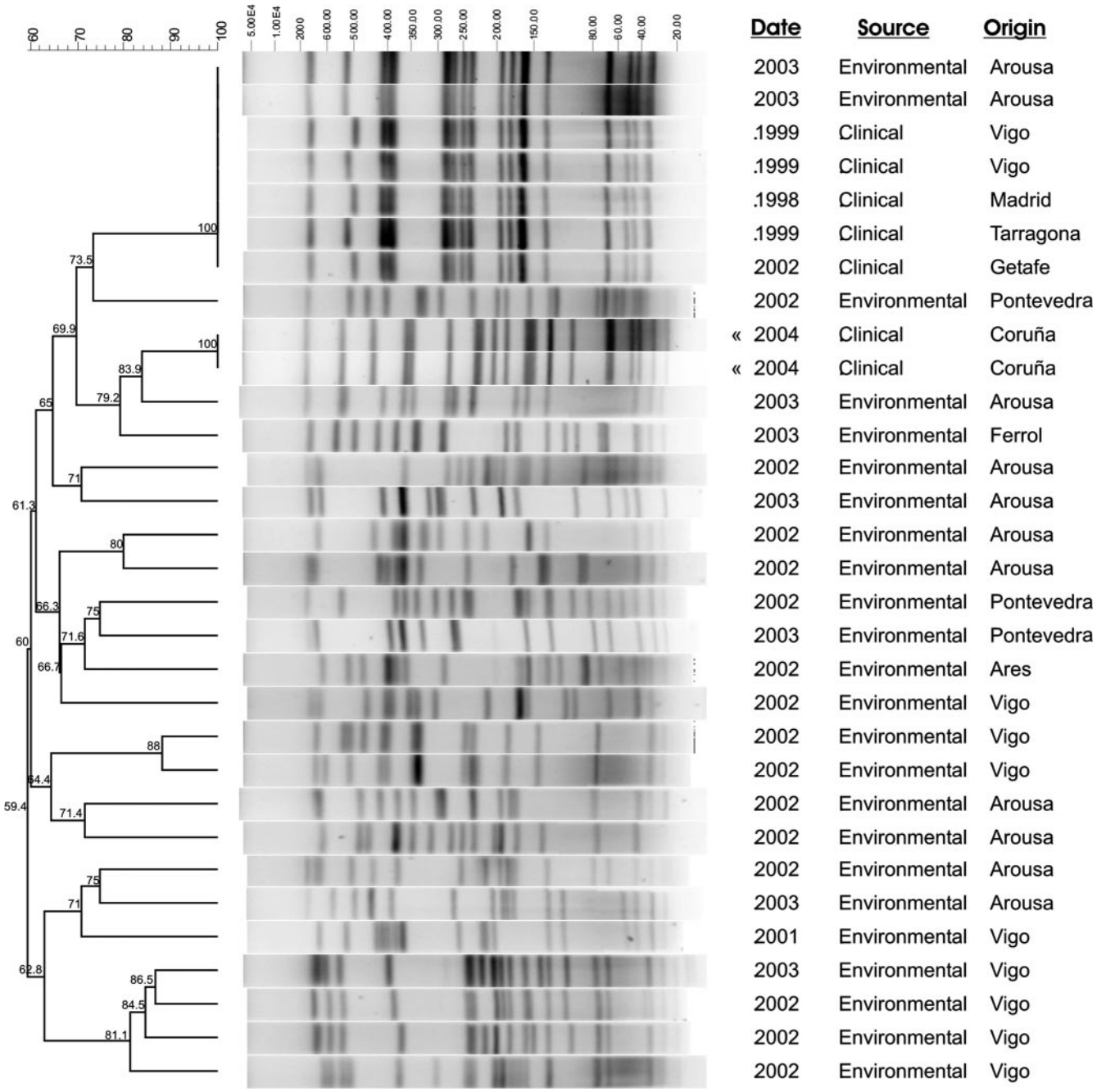

FIG. 5. Dendrogram generated by Bionumeric software showing the relationship of PFGE patterns for *V. parahaemolyticus* isolates included in the comparison. The numbers at the top of the figure indicate molecular sizes in kbp. ≪, pandemic O3:K6 isolates.

marine environments in Galicia, a temperate geographical area with seawater temperatures ranging from 12 to 20°C. Salinity was shown to be the critical factor limiting the temporal and spatial distribution of *V. parahaemolyticus* in the rias of Galicia. The rias are estuarine systems with stable values of salinity (ranging in this study from 30.9 ppt to 36.2 ppt) modulated by a pattern of precipitation that is characterized by heavy rainfall between September and January and moderate precipitation during the rest of the year. *V. parahaemolyticus* was mainly detected during the period of heavy rains in autumn. The highest presence (21%) and abundance (mean value, 1,273 MPN/100 g) of *V. parahaemolyticus* occurred in November, which was also the month of maximum rainfall over the period of study (average, 84 mm/day).

The influence of seawater temperature in the seasonal pattern of *V. parahaemolyticus* was primarily controlled by the effect of salinity. Temperature could modulate the levels of *V. parahaemolyticus* only when warm waters were present during periods of reduced salinity. This pattern of the combined effects of low salinity and warm temperature has also been ob-

served to influence the abundance of *V. parahaemolyticus* in formal investigations carried out in different regions of North America (11, 21). The specific dependence on salinity minimized the presence of *V. parahaemolyticus* during the summer, when the waters were warmest. The low occurrence and abundance of *V. parahaemolyticus* during the summer contrasts with previously reported patterns of presence in other areas of the world, where the highest densities were obtained during the warmest months of summer and low or undetectable levels were observed in autumn and winter (13, 18, 19, 21). The areas of study in previous investigations were mostly confined to estuaries with highly variable values of salinity, ranging from 5 ppt to 30 ppt (9, 12, 20, 21). This range of salinity is probably more suitable for growth of *V. parahaemolyticus*, and under such circumstances, *V. parahaemolyticus* populations are exposed to the influence of temperature over the entire year. Results inferred from the data of this study estimated the maximum probability of detection of *V. parahaemolyticus* at around a salinity of 25 ppt, similar to the levels of salinity reported to enhance the survival, growth, and cellular activity of *Vibrio cholerae* in microcosms (28) and also close to the optimal salinity for *V. parahaemolyticus* abundance in oysters of 23 ppt (13).

The dependence of *V. parahaemolyticus* on the lowest values of salinity was also observed in the pattern of distribution of the organism in the coastal areas of the rias. Results from GIS showed that the gradients of salinity operated as natural boundaries for the presence of *V. parahaemolyticus*. Each ria characteristically has a main river at its inner point that is the primary source of freshwater. The importance of this river, together with the dimensions of each ria, control the circulation of oceanic water into the ria and the levels of salinity in the estuary. Sampling station 7 in the ria of Vigo is located in the interior of the ria, an estuarine area separated from the rest of the ria by a narrow strait that promotes the retention of freshwater from the river and enables relatively high temperatures to be reached due to the reduced inflow of cold oceanic water. These characteristics convert this area into the point of maximum presence of *V. parahaemolyticus* among the rias.

The rias are subjected to seasonal upwelling-downwelling sequences that determine the patterns of circulation and exchange of water in the inner areas of the rias. Upwelling periods predominate in March-April and September-October, while downwelling conditions prevail the rest of the year (1). Results from this study associated the presence of *V. parahaemolyticus* with downwelling periods. During the upwelling season, the continental runoff has a reduced influence on the circulation and the rias show characteristics of coastal upwelling systems rather than of estuaries. By contrast, continental runoff into the rias is important during the downwelling periods, and the rias then resemble estuaries with reduced salinity. Another factor characteristic of downwelling periods is the generation of downwelling fronts through the convergence of salty oceanic water and runoff water with reduced salinity (8). The arrival of oceanic water may promote the influx of zooplankton from the ocean into the interior of the rias and its accumulation in these areas of density transition (26), as has been reported for the accumulation of toxic dinoflagellates in the interior of the rias (29). The presence of patches of zooplankton in the density fronts may lead to the accumulation of *V. parahaemolyticus*, due to the ability of the organism to survive in the marine environment in association with plankton (3, 17).

Molecular characterization of the isolates obtained throughout this study enabled the identification of only two pathogenic isolates of *V. parahaemolyticus*. The extremely low presence of pathogenic populations of *V. parahaemolyticus* in environmental samples is a constant characteristic in most of the investigations carried out in different regions of the world (6, 9, 11, 12, 14, 25). The two isolates identified here are, to our knowledge, the first *tdh*-positive strains of *V. parahaemolyticus* isolated directly from the marine environment of the Atlantic coast of Europe. The presence of pathogenic isolates of *V. parahaemolyticus* has been reported in previous investigations carried out on the coasts of France (16, 27) that identified *trh*-positive strains from marine sources, although no *tdh*-positive isolates were found during these studies. The environmental *tdh*-positive strains obtained from the ria of Arousa showed serotypes and genetic characteristics identical to those of isolates obtained from clinical sources during the outbreaks of infection by *V. parahaemolyticus* recorded in Spain in the same period (22). Most of these clinical strains were obtained from the largest outbreaks of *V. parahaemolyticus* illness detected in Galicia in 1999 and 2000 (22). This finding is the first evidence that identifies the marine environment of Galicia as the source of the pathogenic *V. parahaemolyticus* involved in the large outbreaks of 1999 and 2000.

One factor that may restrict the detection of pathogenic strains is the limitation of the analytical procedures. Analysis of shellfish samples in this study showed an overwhelming dominance of *V. alginolyticus* colonies over *V. parahaemolyticus* populations on TCBS plates. Yellow colonies of *V. alginolyticus* spread rapidly onto TCBS agar, almost completely covering the plates. The dominance of *V. alginolyticus* may have limited the detection of *V. parahaemolyticus* colonies, which were less frequent and grew more slowly. The consecutive streaking of two TCBS plates enhanced the isolation of *Vibrio* species other than *V. alginolyticus* on the plate with the most dilute inoculum and minimized the possibility of false-negative assays during the investigation.

The results of this study reveal the extraordinary importance of salinity in governing the seasonal pattern and the spatial distribution of *V. parahaemolyticus* in the marine environment of the Atlantic coast of Europe. This information provides a novel perspective regarding the ecological conditions regulating the dynamic of *V. parahaemolyticus* in temperate regions and is of practical use in managing shellfish-harvesting areas.

ACKNOWLEDGMENTS

We are grateful to the staff of the Unidad de Control de Moluscos Laboratory (Instituto de Acuicultura, Universidad de Santiago de Compostela), Juan Ansede Bermejo, and Veronica Blanco Abad for technical assistance.

REFERENCES

1. **Alvarez-Salgado, X. A., J. Gago, B. M. Miguez, M. Gilcoto, and F. F. Perez.** 2000. Surface waters of the NW Iberian margin: upwelling on the shelf versus outwelling of upwelled waters from the Rias Baixas. Estuar. Coast Shelf Sci. **51:**821–837.
2. **Anonymous.** 2001. Opinion of the scientific committee on veterinary measures relating to public health on *Vibrio vulnificus* and *Vibrio parahaemolyticus*. European Commission, Brussels, Belgium.

3. **Baffone, W., R. Tarsi, L. Pane, R. Campana, B. Repetto, G. L. Mariottini, and C. Pruzzo.** 2006. Detection of free-living and plankton-bound vibrios in coastal waters of the Adriatic Sea (Italy) and study of their pathogenicity-associated properties. Environ. Microbiol. **8:**1299–1305.
4. **Bej, A. K., D. P. Patterson, C. W. Brasher, M. C. Vickery, D. D. Jones, and C. A. Kaysner.** 1999. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of tl, tdh and trh. J. Microbiol. Methods **36:**215–225.
5. **Cabello, F. C., R. Espejo, M. C. Hernandez, M. R. Rioseco, J. Ulloa, and J. A. Vergara.** 2007. *Vibrio parahaemolyticus* O3:K6 epidemic diarrhea, Chile, 2005. Emerg. Infect. Dis. **13:**655–656.
6. **Cabrera-Garcia, M. E., C. Vazquez-Salinas, and E. I. Quinones-Ramirez.** 2004. Serologic and molecular characterization of *Vibrio parahaemolyticus* strains isolated from seawater and fish products of the Gulf of Mexico. Appl. Environ. Microbiol. **70:**6401–6406.
7. **CDC.** 2002. Standardized molecular subtyping of foodborne bacterial pathogens by pulsed-field gel electrophoresis. Centers for Diseases Control and Prevention, Atlanta, GA.
8. **Crespo, B. G., F. G. Figueiras, P. Porras, and I. G. Teixeira.** 2006. Downwelling and dominance of autochthonous dinoflagellates in the NW Iberian margin: the example of the Ria de Vigo. Harmful Algae **5:**770–781.
9. **Deepanjali, A., H. S. Kumar, I. Karunasagar, and I. Karunasagar.** 2005. Seasonal variation in abundance of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters along the southwest coast of India. Appl. Environ. Microbiol. **71:**3575–3580.
10. **DePaola, A., and C. Kaisner.** 2001. *Vibrio*, p. 405–428. *In* F. P. Downes and K. Ito (ed.), Microbiological examination of foods. American Public Health Association, Washington, DC.
11. **DePaola, A., C. A. Kaysner, J. Bowers, and D. W. Cook.** 2000. Environmental investigations of *Vibrio parahaemolyticus* in oysters after outbreaks in Washington, Texas, and New York (1997 and 1998). Appl. Environ. Microbiol. **66:**4649–4654.
12. **DePaola, A., J. L. Nordstrom, J. C. Bowers, J. G. Wells, and D. W. Cook.** 2003. Seasonal abundance of total and pathogenic *Vibrio parahaemolyticus* in Alabama oysters. Appl. Environ. Microbiol. **69:**1521–1526.
13. **FDA.** 2005. Quantitative risk assessment on the public health impact of pathogenic *Vibrio parahaemolyticus* in raw oysters. FDA, Washington, DC.
14. **Fuenzalida, L., C. Hernandez, J. Toro, M. L. Rioseco, J. Romero, and R. T. Espejo.** 2006. *Vibrio parahaemolyticus* in shellfish and clinical samples during two large epidemics of diarrhoea in southern Chile. Environ. Microbiol. **8:**675–683.
15. **Gonzalez-Escalona, N., V. Cachicas, C. Acevedo, M. L. Rioseco, J. A. Vergara, F. Cabello, J. Romero, and R. T. Espejo.** 2005. *Vibrio parahaemolyticus* diarrhea, Chile, 1998 and 2004. Emerg. Infect. Dis. **11:**129–131.
16. **Hervio-Heath, D., R. R. Colwell, A. Derrien, A. Robert-Pillot, J. M. Fournier, and M. Pommepuy.** 2002. Occurrence of pathogenic vibrios in coastal areas of France. J. Appl. Microbiol. **92:**1123–1135.
17. **Joseph, S. W., R. R. Colwell, and J. B. Kaper.** 1982. *Vibrio parahaemolyticus* and related halophilic vibrios. Crit. Rev. Microbiol. **10:**77–124.
18. **Kaneko, T., and R. R. Colwell.** 1978. Annual cycle of *Vibrio parahaemolyticus* in Chesapeake Bay. Microb. Ecol. **4:**135–155.
19. **Kaneko, T., and R. R. Colwell.** 1973. Ecology of *Vibrio parahaemolyticus* in Chesapeake Bay. J. Bacteriol. **113:**24–32.
20. **Kaneko, T., and R. R. Colwell.** 1975. Incidence of *Vibrio parahaemolyticus* in Chesapeake Bay. Appl. Microbiol. **30:**251–257.
21. **Kelly, M. T., and E. M. D. Stroh.** 1988. Temporal relationship of *Vibrio parahaemolyticus* in patients and the environment. J. Clin. Microbiol. **26:** 1754–1756.
22. **Martinez-Urtaza, J., A. Lozano-Leon, A. DePaola, M. Ishibashi, K. Shimada, M. Nishibuchi, and E. Liebana.** 2004. Characterization of pathogenic *Vibrio parahaemolyticus* isolates from clinical sources in Spain and comparison with Asian and North American pandemic isolates. J. Clin. Microbiol. **42:**4672–4678.
23. **Martinez-Urtaza, J., L. Simental, D. Velasco, A. DePaola, M. Ishibashi, Y. Nakaguchi, M. Nishibuchi, D. Carrera-Flores, C. Rey-Alvarez, and A. Pousa.** 2005. Pandemic *Vibrio parahaemolyticus* O3:K6, Europe. Emerg. Infect. Dis. **11:**1319–1320.
24. **McLaughlin, J. B., A. DePaola, C. A. Bopp, K. A. Martinek, N. P. Napolilli, C. G. Allison, S. L. Murray, E. C. Thompson, M. M. Bird, and J. P. Middaugh.** 2005. Outbreak of *Vibrio parahaemolyticus* gastroenteritis associated with Alaskan oysters. N. Engl. J. Med. **353:**1463–1470.
25. **Nair, G. B., T. Ramamurthy, S. K. Bhattacharya, B. Dutta, Y. Takeda, and D. A. Sack.** 2007. Global dissemination of *Vibrio parahaemolyticus* serotype O3:K6 and its serovariants. Clin. Microbiol. Rev. **20:**39.
26. **Olson, D. B., G. L. Hitchcock, A. J. Mariano, C. J. Ashjan, G. Peng, R. W. Nero, and G. P. Podesta.** 1994. Life on the edge: marine life and fronts. Oceanography **7:**52–60.
27. **Robert-Pillot, A., A. Guenole, J. Lesne, R. Delesmont, J. M. Fournier, and M. L. Quilici.** 2004. Occurrence of the *tdh* and *trh* genes in *Vibrio parahaemolyticus* isolates from waters and raw shellfish collected in two French coastal areas and from seafood imported into France. Int. J. Food Microbiol. **91:**319–325.
28. **Singleton, F. L., R. Attwell, S. Jangi, and R. R. Colwell.** 1982. Effects of temperature and salinity on *Vibrio cholerae* growth. Appl. Environ. Microbiol. **44:**1047–1058.
29. **Tilstone, G. H., F. G. Figueiras, and F. Fraga.** 1994. Upwelling-downwelling sequences in the generation of red tides in a coastal upwelling system. Mar. Ecol. Prog. Ser. **112:**241–253.