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# Occurrence, seasonality and infectivity of *Vibrio* strains in natural populations of mussels *Mytilus galloprovincialis*

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ABSTRACT: Widespread and large-scale mortalities of bivalve molluscs significantly affect their production. A number of pathogens have been identified as the primary causes of death in oysters or clams, especially bacteria of the genus *Vibrio*. We evaluated the occurrence, seasonality and infectivity of *Vibrio* strains associated with natural mussel (*Mytilus galloprovincialis*) populations. In particular, different isolates of *V. splendidus* and *V. aestuarianus* were analysed because they were associated with major oyster mortalities in areas where mussels are cultured without presenting mortalities. The presence of both *Vibrio* spp. was analysed bimonthly in mussels, water, sediment, plankton and other associated fauna from 2 sites in Galicia (NW Spain), the region with the highest mussel production in Europe. Environmental factors were also considered. The pathogenicity of different *Vibrio* isolates was analysed by performing experimental infections in mussels with strains isolated from the field. Results showed that *Vibrio* populations were mainly influenced by changes in water temperature and salinity. *V. splendidus* was dominant during the warm months and *V. aestuarianus* was predominant throughout the cold season. The sediment was the most important natural reservoir for bacteria. Experimental infections showed the extreme resistance of mussels to bacterial pathogens. Isolates of *V. splendidus* and *V. aestuarianus* were only moderately pathogenic for mussels in intramuscular infections and bath infections, and mortalities only occurred when animals were infected with a high bacterial concentration in adverse environmental conditions (hypoxia and 25°C). Although the pathogenicity of the *Vibrio* strains isolated from the wild was low for mussels, their potential risk for other bivalves cannot be ignored.

KEY WORDS: Pathogenicity · Environmental conditions · Natural reservoirs · *Crassostrea gigas* · Bivalve · Infection model



## INTRODUCTION

The Mediterranean mussel *Mytilus galloprovincialis* and the Pacific oyster *Crassostrea gigas* are the 2 most important bivalve species in European aquaculture. Spain is the leading European producer of the Mediterranean mussel, and France is the primary producer of the Pacific oyster (FAO 2013).

Disease-associated mortalities in these 2 important species can result in significant loses with major economic consequences. Aetiological disease agents in farmed bivalves, such as *Bonamia*, *Haplosporidium*, *Perkinsus*, *Marteilia*, several strains of *Vibrio* and also herpesviruses have been characterized after the occurrence of massive mortality outbreaks (Figueras & Novoa 2011). Deaths in bivalves are associated

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with unusual changes in temperature, salinity or pollution (Tsuchiya 1983, Pipe & Coles 1995, Aranguren et al. 2012), as well as with normal seasonal fluctuations of those environmental parameters (Degremont et al. 2005, Soletchnik et al. 2007).

Bacteria belonging to the genus *Vibrio* are autochthonous to aquatic environments, and some species such as *V. anguillarum* biovar I, *V. splendidus, V. tapetis, V. aestuarianus* or *V. harveyi* are pathogens of marine organisms, including fish, corals and molluscs (Nicolas et al. 1996, Thompson et al. 2004, Toranzo et al. 2005, Beaz-Hidalgo et al. 2010). Vibriosis in larval-stage bivalve molluscs in hatcheries has been widely reported (Jeffries 1982, Nicolas et al. 1996, Sugumar et al. 1998, Waechter et al. 2002, Gómez-León et al. 2005). In contrast, with the exception of brown ring disease in Manila clam *Ruditapes philippinarum* (Figueras et al. 1996, Allam et al. 2002), fewer studies have reported vibriosis in juvenile and adult molluscs. Two pathogenic strains of *V. splendidus* were identified in the Pacific oyster *Crassostrea gigas* (Lacoste et al. 2001, Le Roux et al. 2002, Waechter et al. 2002, Gay et al. 2004a,b), and were associated with summer mortalities (Garnier et al. 2007). *V. splendidus* and related species were also detected in *Chamelea gallina* (Torresi et al. 2011). Other *Vibrio* species, such as *V. aestuarianus,* cause mortalities in Pacific oyster (Garnier et al. 2008, Azandégbé et al. 2010, Labreuche et al. 2010), and *V. harveyi* has also been associated with mass mortality episodes in the abalone *Haliotis tuberculata* (Nicolas et al. 2002).

A number of studies have highlighted the importance of natural reservoirs, where pathogenic microorganisms can persist before being released to cause an infection (Colwell 1984, Covazzi Harriague et al. 2008, Turner et al. 2009, Vezzulli et al. 2009, 2010, Azandégbé et al. 2010). However, no information is available regarding the prevalence and pathogenicity of *Vibrio splendidus* and *V. aestuarianus* in natural mussel populations. These pathogens could pose a risk to bivalve production, and mussels might provide a niche for the proliferation and distribution of these bacteria. Therefore, it is necessary to determine the prevalence, seasonality and infectivity of *V. splendidus* and *V. aestuarianus* associated with natural mussel populations, since an emergence of pathogenic *Vibrio* spp. in coastal zones has been predicted based on the unusual increase in seawater temperature (Marcogliese 2008, Martinez-Urtaza et al. 2008, Vezzulli et al. 2012).

In the present work, the distribution and prevalence of *Vibrio splendidus* and *V. aestuarianus* were determined at 2 locations in Galicia (NW Spain) in natural mussel populations and in different ecological compartments to establish their implications as reservoirs for these pathogens. Classical culture-dependent and culture-independent methods were used to explore the ecology and distribution of *Vibrio* spp. in aquatic environments.

Vibriosis-induced mortalities and the mechanism and regulation of the host immune response in adult bivalves are still poorly understood. The potential risks of the different isolates of *Vibrio splendidus* and *V. aestuarianus* to the mussel industry were analysed using experimental infections. Our study provides novel insight into *Vibrio* pathogens and their interactions with environmental factors that could affect mussel production.

## MATERIALS AND METHODS

### Sampling and collection of environmental and biological samples

The 2 different locations for sampling in Galicia (NW Spain) were Alcabre (42° 13′ 25″ N, 8° 45′ 37″ W) and the Sor River (43° 42′ 59″ N, 7° 41′ 48″ W). No sampling stations were considered environmental protected areas (Fig. 1). Six samplings were conducted every 2 mo for 1 yr, from June 2011 to April 2012. Water temperature, salinity and pH were recorded in the field using portable devices. Samples of water, surface sediment (0–2 cm) and plankton were taken for bacterial analysis when the tidal level was low (0.5 m). Plankton was collected by dragging a 200 µm nylon mesh horizontally across the water surface (100 m at 0–30 cm depth). Environmental samples were taken from exactly the same sampling point as animals. A total of 30 mussels (mean length ± SD = 4.8 ± 0.29 cm) were taken from each location, and 30 adult oysters were also collected from the Sor River. Small animals of the most abundant taxonomic groups of fauna associated with mussels (cnidarians, molluscs, annelids and crustaceans) were also sampled. No specific permits were required for the described field studies, as they did not involve endangered species.

### Sample processing for molecular and microbiological analysis

Water samples (500 ml) were filtered through a 200 µm mesh to remove particulate material and then

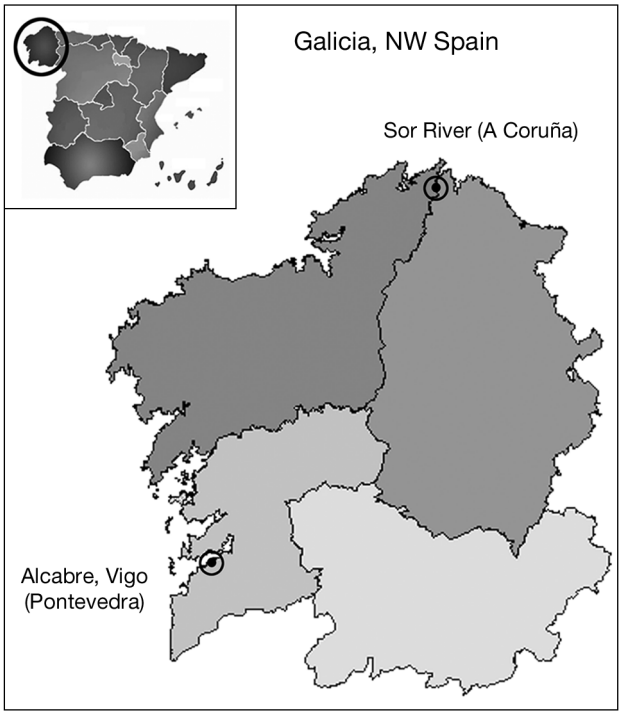


Fig. 1. Location of the 2 sampling points, Alcabre and the Sor River, in Galicia (NW Spain)

re-filtered through a 0.22 µm membrane. The bacteria retained by the filter were resuspended in 100 ml sterile-filtered seawater (FSW). Aliquots (0.5 ml) were removed for bacteriology, the remaining volume was centrifuged at 4000 × *g* (10 min at 4 °C), and the pellets were kept for DNA isolation. Sediment samples (5 g) were resuspended in 50 ml FSW and vortexed. Aliquots were removed for bacteriology, and additional sediment samples (1 g) were reserved for DNA isolation. The plankton suspension was centrifuged at 1000 × *g* (10 min) to pellet the plankton organisms. A 1 g aliquot was resuspended in 10 ml FSW for microbiological analysis, and an additional 1 g was used for DNA isolation.

Bivalve tissues (gills, mantle and digestive gland) were extracted. For total bacterial quantification, sections of the same tissue from 5 animals were pooled (total weight: 25–40 mg) and homogenised in FSW (1:1, w/v). To isolate and identify different strains of *Vibrio* spp., organs were homogenised and diluted 1:100 and 1:10 000 in FSW. Tissues were also used for total DNA isolation. Associated macrofauna were identified. Individuals that belonged to the same taxonomic group were pooled and fixed in absolute alcohol at room temperature. DNA isolated from each different sample corresponded to the same taxonomic group.

Total bacterial quantification was conducted in tryptone soya agar (TSA) supplemented with NaCl (1.5%). For *Vibrio* spp., environmental samples (0.5 ml) were enriched in 4.5 ml alkaline peptone water (APW, final pH 8.6) for 24 h at 25°C and spread onto thiosulphate-citrate-bile-sucrose agar (TCBS) plates. Homogenised animal tissue suspensions were directly cultured on TCBS plates. Individual colonies with distinct morphology were selected and identified by qPCR (described in the next section). The different isolates of *Vibrio* spp. were also identified using DNA isolated directly from environmental samples (water, sediment and plankton) and from animal tissues following the protocol described in the next section. The fluctuation of total bacterial load as a function of the environmental conditions was assayed by Pearson's R correlation test.

### DNA isolation and *Vibrio* detection by qPCR

Total DNA from animal tissues and macro-fauna was isolated using the LEV Blood DNA kit (Promega). DNA was isolated from water using the High Pure Polymerase Chain Reaction Template Preparation Kit (Roche Diagnostics). The DNA from sediment and plankton was isolated using the UltraClean Soil DNA kit (MO BIO Laboratories). DNA was isolated from individual colonies grown on TCBS plates by resuspending them into 200 µl sterile water and heating them at 100°C for 20 min.

Quantitative PCR was performed on an MX3000 Thermocycler (Stratagene) with specific probes using the Brilliant III 2X Ultra-Fast Master Mix (Agilent). The PCR conditions and the probe and primer sequences for *Vibrio aestuarianus* and *V. splendidus* were described by Saulnier et al. (2009) and by the European Reference Laboratory for Mollusc Diseases (IFREMER, France; www.eurl-mollusc.eu/News/Quality-management-and-SOPs). Each sample was tested in duplicate and was considered positive if its $C_t$ value was below 37 and the difference between duplicated values did not exceed 0.5 $C_t$. Positive and negative controls for qPCR were included in each reaction.

Standard curves were obtained to quantify *Vibrio splendidus* and *V. aestuarianus* in animal tissue. The standards from pure cultures of *V. splendidus* (LGP-32 strain; Gay et al. 2004a) and *V. aestuarianus* (01/032 strain; Garnier et al. 2008) were grown at 25°C in TSB+1.5% NaCl for 18 h and serially diluted 1:10 in FSW. The bacterial concentration was calculated using TSA+1.5% NaCl plates. Duplicated samples of

each serial dilution (200 µl) were also used for DNA extraction. DNA extracts were analysed in triplicate by qPCR. Pearson's correlation coefficients ($R^2$) and simple linear regression equations were calculated to correlate the qPCR ($C_t$) and the traditional culture-based method (CFU $ml^{-1}$) results.

### PCR amplification and sequencing the 16S rRNA gene

*Vibrio* isolates that were not identified by qPCR (not *V. splendidus* or *V. aestuarianus*) were identified using the methodology previously described by Campbell et al. (1995). Briefly, the bacterial 16S ribosomal RNA was amplified by PCR using the PSL primers. The PCR products were cloned and sequenced. ChromasPro (v.1.33) software was used for sequence analysis. Subsequently, the sequences were examined using the BlastN alignment tool to determine which bacterial group in GenBank was closest to each strain. The obtained sequences were deposited in GenBank (accession numbers: KF-170564–KF170735).

### Experimental infections of mussels

Experimental disease models were developed to explore the susceptibility of mussels to *Vibrio* infection. Two different infection protocols were used: bath exposure and intramuscular injection.

Challenges were conducted by bath exposure using a total of 540 animals (mean length ± SD = 2.08 ± 0.4 cm) distributed among 36 tanks (15 animals $tank^{-1}$), and performing 4 replicates for each treatment. Animals were immersed in FSW containing $10^{10}$ CFU $ml^{-1}$ *Vibrio splendidus* or *V. aestuarianus.* A high bacterial dose was used to induce mortalities in mussels. The volume of the tanks was 2 l, and the seawater was exchanged 3 times $wk^{-1}$ during the experimental infection. Animals were fed daily with a mixture of different microalgae during the entire experiment. To analyse the effect of environmental conditions on mortality, infection experiments were conducted in parallel at 15 and 25°C. Each day, half of the infected animals at each temperature were subjected to an 8 h emersion to mimic the natural effect of the tides (hypoxia-induced stress). Cumulative mortality was monitored for 15 d.

Acute infections were generated via intramuscular injection at 15°C. This temperature was selected since it is close to the annual average (±SD) value (13.9 ± 4.4°C) that we recorded. A total of 420 mussels (2.75 ± 0.4 cm long) were distributed into groups of 20. A total of 21 tanks containing 1 l of seawater were used. For the inoculation, reference bacteria (*Vibrio splendidus* strain LGP32 and *V. aestuarianus* strain 01/032) were grown for 12 h in TSA+1.5% NaCl plates and resuspended in FSW. We prepared 100 µl of 3 different doses of *V. splendidus* and *V. aestuarianus* in FSW ($10^9$, $10^8$ and $10^7$ CFU $ml^{-1}$) and injected them into the posterior adductor muscle. The control group was injected with FSW. Three replicates were generated for each treatment, and cumulative mortality was recorded for 10 d. Animals were maintained as previously described.

### Experimental infections of mussels with *Vibrio* strains isolated from the field

The susceptibility of mussels to all *Vibrio* strains isolated from the field was studied by intramuscular injection of 390 animals (3.36 ± 0.67 cm long). Seven isolates of *V. splendidus* and 3 isolates of *V. aestuarianus* were studied. Reference strains of *V. splendidus* (LGP32) and *V. aestuarianus* (01/032) were used at the same dose. Animals (15 in each group) were injected on the posterior adductor muscle at 15°C with 100 µl of the different isolates at a dose of $10^{11}$ CFU $ml^{-1}$. The control group was injected with FSW. Three replicates were performed for each treatment. Cumulative mortality was monitored for 10 d. Animals were maintained in 1 l aquariums as previously described.

## RESULTS

### Environmental parameters

In Alcabre, salinity ranged from 33 ppt in October to 42 ppt in February. The salinity of the Sor River showed greater variability, fluctuating from 35 ppt in August to a low of 14 ppt in April. Water temperature decreased during winter, with the lowest values in February (11 and 9°C in Alcabre and the Sor River, respectively). pH was constant at both locations (Fig. 2).

### Bacterial concentration

In Alcabre, the total cultural bacteria concentration in water and sediment reached a maximum in August ($5 \times 10^6$ CFU $ml^{-1}$ and $2 \times 10^4$ CFU $g^{-1}$, respectively).

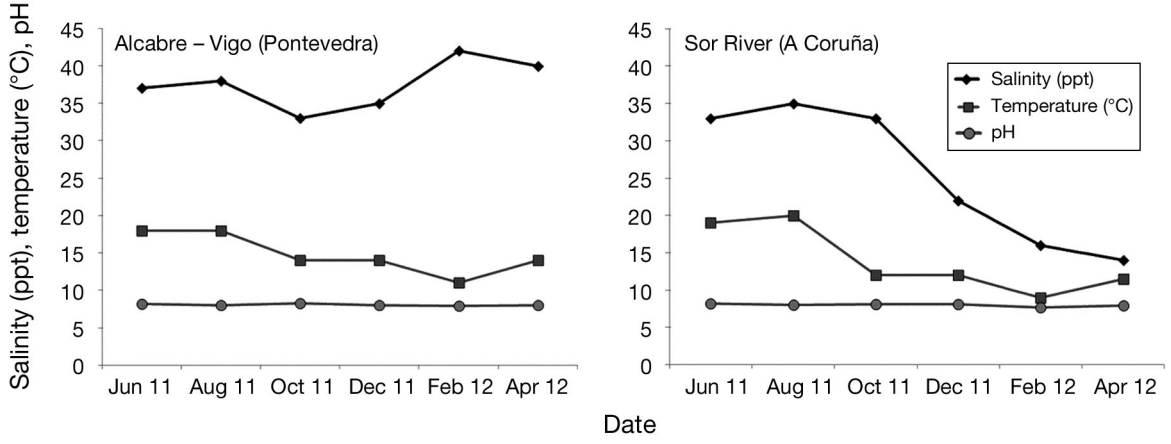


Fig. 2. Changes in environmental parameters (salinity, water temperature and pH) from June 2011 to April 2012 in Alcabre and the Sor River

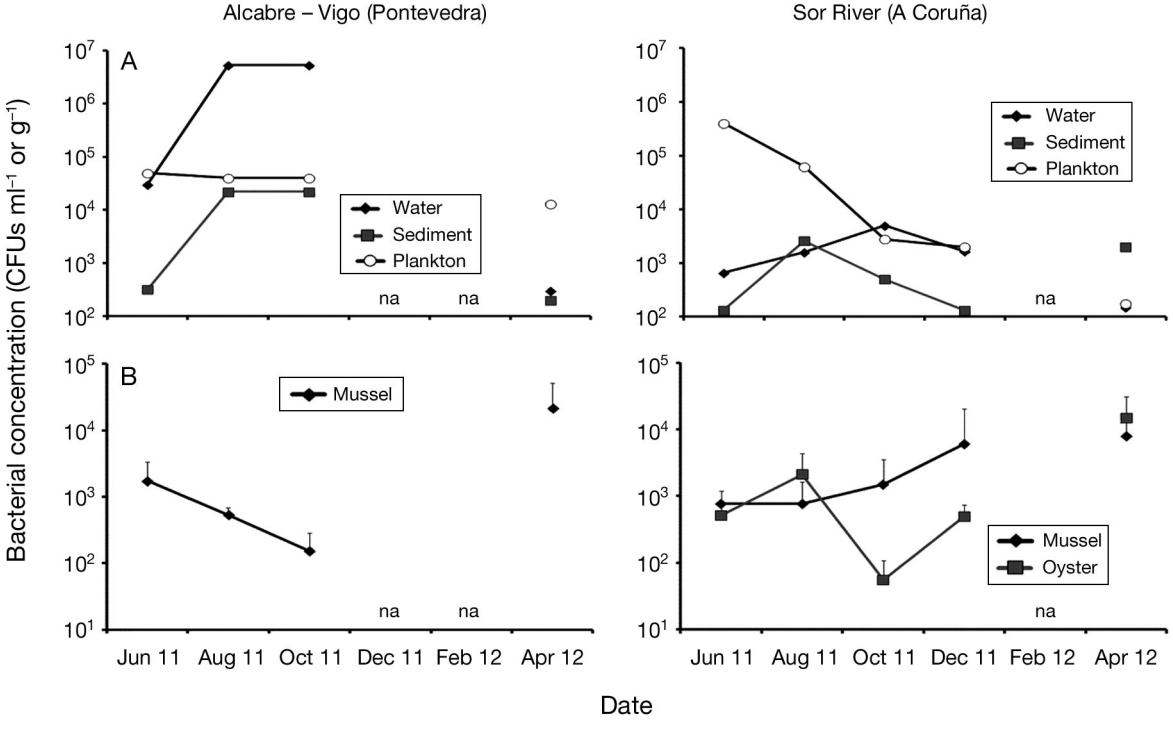


Fig. 3. Total bacterial concentration in (A) environmental samples (water, sediment and plankton) and in (B) mussels and oysters from Alcabre and the Sor River. na: sample not analysed

The bacterial load decreased until reaching a low in April ($3 \times 10^2$ CFU ml$^{-1}$ and $2 \times 10^2$ CFU g$^{-1}$ in water and sediment, respectively). We cannot be certain that this was the lowest bacterial load since samples from December and February were not analysed. The total bacterial concentration in plankton ($1 \times 10^4$ CFU g$^{-1}$) remained constant throughout the year (Fig. 3A).

In the Sor River, the bacterial concentrations were lower than those recorded in Alcabre. In water, the maximum concentration occurred in October ($5 \times 10^3$ CFU ml$^{-1}$), and the concentration decreased until the end of the sampling period. The bacterial load in sediment was highest in August ($2.6 \times 10^3$ CFU g$^{-1}$) and lowest in winter ($1.3 \times 10^2$ CFU g$^{-1}$). For the plankton samples, the bacterial levels decreased at each sampling point from $4 \times 10^5$ CFU g$^{-1}$ in June to $1.8 \times 10^2$ CFU g$^{-1}$ in April (Fig. 3A).

The total bacterial concentration was also measured in mussels from Alcabre and in mussels and oysters from the Sor River (Fig. 3B). In Alcabre, the bacterial load increased during the warm months, reaching a maximum in April (Fig. 3B). Samples from the Sor River had similar kinetics, peaking in April at $7.9 \times 10^3$ and $1.4 \times 10^4$ CFU $ml^{-1}$ $g^{-1}$ in mussels and oysters, respectively (Fig. 3B). Pearson's R correlations indicated that salinity was the most important factor influencing the total bacterial load in samples from Alcabre (R > 0.7), whereas changes in salinity and temperature influenced the bacterial load in the Sor River (R values ranging from 0.6 to 0.9).

### Detection of *Vibrio splendidus* and *V. aestuarianus* in animals and environmental samples

The qPCR results for the detection and quantification of *V. splendidus* and *V. aestuarianus* in animals by qPCR using DNA directly extracted from tissue are presented in Fig. 4. Mussels collected from Alcabre (Fig. 4A) and the Sor River (Fig. 4B) were similar regarding the presence of both *Vibrio* spp. In summer, a high percentage of animals were apparently healthy carriers of *V. splendidus* (76.6% and 63.3% of the mussels from Alcabre and the Sor River, respectively). At that time, the percentage of animals positive for *V. aestuarianus* was less than 13%. In October and December, the prevalence of *V. splendidus* significantly decreased in animals from Alcabre, and *V. splendidus* was only present in 3% of the animals. Over the same period, the percentage of healthy carrier animals with *V. aestuarianus* rose to 66% in Alcabre and 86% in the Sor River. Between February and April, the incidence of *V. splendidus* increased to >70%, and that of *V. aestuarianus* dropped to 15% (Fig. 4A,B). *V. aestuarianus* was not detected in mussels from Alcabre in February 2012 or from the Sor River in August 2011. The results indicated that *V. splendidus* and *V. aestuarianus* could coexist in the same animal. The highest percentage of doubly infected mussels was observed in October, with values of 20% in Alcabre and 46% in the Sor River (Fig. 4A,B).

A statistically significant linear correlation existed between the qPCR and the traditional culture-based *Vibrio* spp. quantification results. The $R^2$ values for the standard curves were 0.98 and 0.99. The evolution of the bacterial load in an animal followed the same kinetics as the prevalence: the higher the percentage of infected animals, the greater the tissue concentration of *Vibrio* spp. (Fig. 4A,B). In August, numerous mussels were infected with stains of *V. splendidus*, and the bacterial load was high ($2 \times 10^2$ CFU $ml^{-1}$). At this time, the number and bacterial load of animals infected with *V. aestuarianus* were low ($5 \times 10^{-2}$ CFU $ml^{-1}$). In February, the highest percentage of mussels infected with *V. aestuarianus* and the highest bacterial load were observed at the Sor River ($6.3 \times 10^1$ CFU $ml^{-1}$; Fig. 4A,B).

In the Sor River, the prevalence of *Vibrio splendidus* in oysters was high throughout the year (between 36 and 76%), and few animals were infected with *V. aestuarianus* (less than 17%). The number of doubly infected animals was low (Fig. 4C). *V. aestuarianus* was not detected in oysters collected in June 2011, August 2011 or April 2012.

Similar percentages of detection of *Vibrio splendidus* were registered in all organs (gills, mantle and digestive gland). In contrast, *V. aestuarianus* was primarily detected in the mantle, regardless of the sampling point and the species. The percentages of detection of *V. aestuarianus* were significantly higher in mantle samples than in gills and digestive gland (Fig. 4D).

The bacterial DNA results from water, sediment, plankton and associated macrofauna are presented in Table 1. Strains of *Vibrio aestuarianus* were not detected at any sampling location or date. Strains of *V. splendidus* were detected in a few environmental samples but were present in almost all of the associated macrofauna samples. In Alcabre, water, plankton and sediment samples were positive in December and February. In the Sor River, only the plankton collected in April was positive (Table 1).

### Identification of *Vibrio* isolates from TCBS plates

Only 80 out of 292 bacteria grown in TCBS were successfully amplified with the specific probes for *V. aestuarianus* and *V. splendidus*. In Alcabre and the Sor River, *V. splendidus* was detected from October to April in all of the environmental samples, but *V. aestuarianus* was never observed in Alcabre. In mussel and oyster tissue and plankton from the Sor River, a small percentage of the analysed bacteria were identified as *V. aestuarianus* (only 3 out of 80 identified bacteria; Table 2).

Bacteria that grew on TCBS plates, but were not identified by qPCR (i.e. were not *Vibrio splendidus* or *V. aestuarianus*) were classified by 16S rRNA gene amplification and sequencing. A total of 212 bacterial strains were analysed. The representation of each

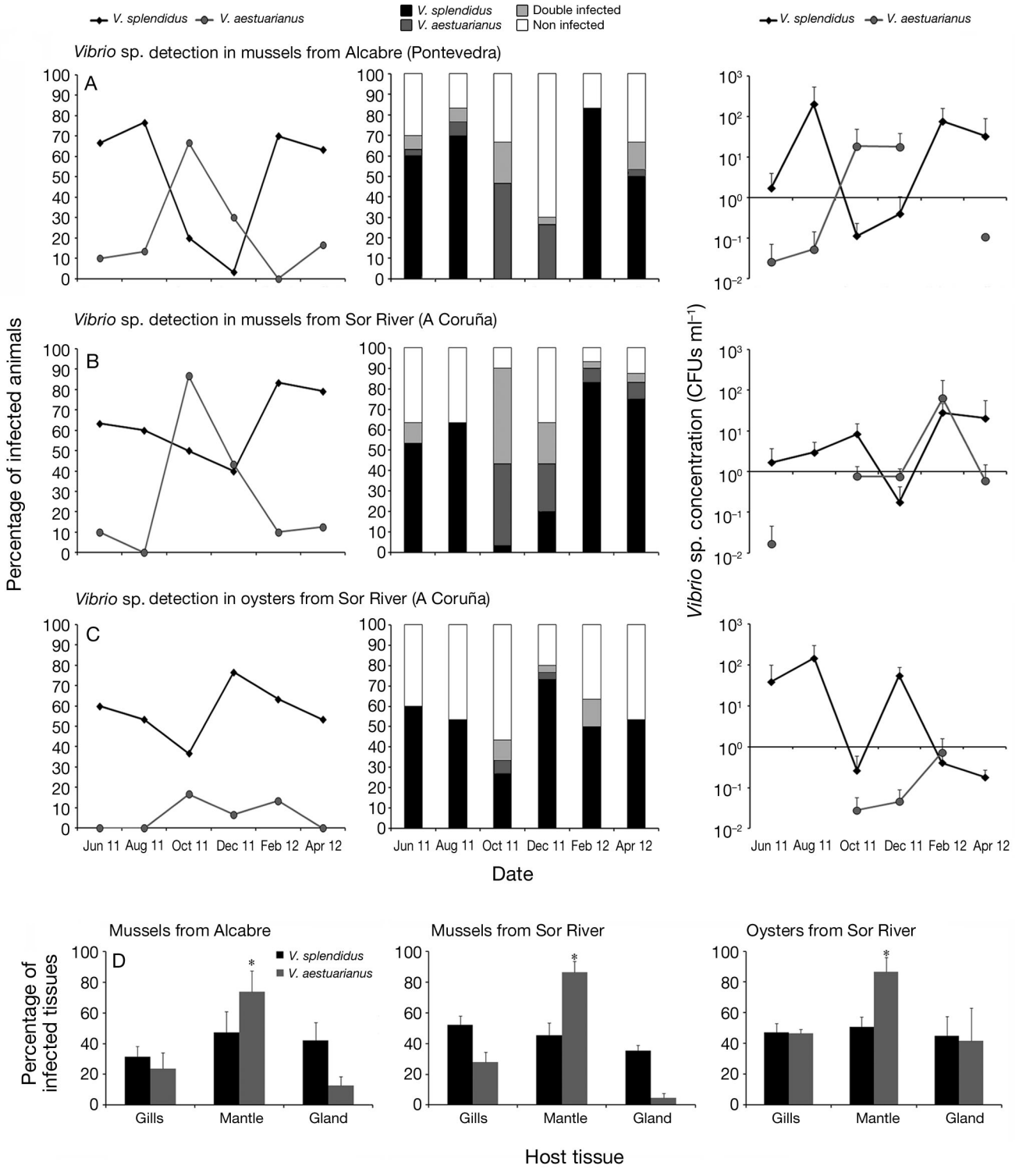


Fig. 4. Quantification of *Vibrio* spp. by qPCR using DNA extracted from mussels from (A) Alcabre and (B) the Sor River and (C) oysters from the Sor River during the study year. The bars represent the proportion of animals infected with 1 type of *Vibrio*, doubly infected animals and non-infected animals. The right panels represent the bacterial concentration (mean + SD) in tissue samples from mussels and oysters calculated using a standard. (D) Distribution of *V. splendidus* and *V. aestuarianus* in the infected tissues of mussels taken from Alcabre and the Sor River and of oysters from the Sor River. Data are means + SEM of the percentage of infected animals in 1 yr. (*) significant differences compared to results obtained in gills and digestive gland ($p \geq 0.05$)

Table 1. Detection of *Vibrio splendidus* (*V. spl*) and *V. aestuarianus* (*V. aest*) in environmental samples and macrofauna by qPCR. (+) positive detection; (–) negative detection; NA: not analysed

| Source | June 2011 | | August 2011 | | October 2011 | | December 2011 | | February 2012 | | April 2012 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *V. spl* | *V. aest* | *V. spl* | *V. aest* | *V. spl* | *V. aest* | *V. spl* | *V. aest* | *V. spl* | *V. aest* | *V. spl* | *V. aest* |
| **Alcabre** | | | | | | | | | | | | |
| Water | – | – | – | – | – | – | + | – | – | – | – | – |
| Sediment | – | – | – | – | – | – | – | – | + | – | – | – |
| Plankton | – | – | – | – | – | – | + | – | – | – | – | – |
| Cnidaria | + | – | – | – | NA | NA | NA | NA | + | – | + | – |
| Gastropoda | – | – | – | – | – | – | – | – | – | – | NA | NA |
| Bivalvia | NA | NA | – | – | + | – | – | – | NA | NA | NA | NA |
| Polychaeta | + | – | – | – | – | – | – | – | – | – | + | – |
| Decapoda | + | – | – | – | + | – | – | – | – | – | NA | NA |
| Cirripeda | NA | NA | + | – | + | – | NA | NA | – | – | + | – |
| Amphipoda | NA | NA | + | – | NA | NA | NA | NA | NA | NA | NA | NA |
| **Sor River** | | | | | | | | | | | | |
| Water | – | – | – | – | – | – | – | – | – | – | – | – |
| Sediment | – | – | – | – | – | – | – | – | – | – | – | – |
| Plankton | – | – | – | – | – | – | – | – | – | – | + | – |
| Cnidaria | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| Gastropoda | – | – | – | – | – | – | – | – | – | – | – | – |
| Bivalvia | + | – | – | – | + | – | – | – | + | – | + | – |
| Polychaeta | + | – | – | – | + | – | – | – | – | – | – | – |
| Decapoda | – | – | – | – | + | – | + | – | – | – | + | – |
| Cirripeda | NA | NA | NA | NA | – | – | + | – | NA | NA | – | – |
| Amphipoda | – | – | – | – | NA | NA | NA | NA | NA | NA | NA | NA |

Table 2. Percentage of positive colonies, previously grown in thiosulphate-citrate-bile-sucrose agar (TCBS), identified by specific qPCR as *Vibrio splendidus* (*V. spl*) or *V. aestuarianus* (*V. aest*). (–) absence of colony growth in TCBS plates

| Source | June 2011 | | August 2011 | | October 2011 | | December 2011 | | February 2012 | | April 2012 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *V. spl* | *V. aest* | *V. spl* | *V. aest* | *V. spl* | *V. aest* | *V. spl* | *V. aest* | *V. spl* | *V. aest* | *V. spl* | *V. aest* |
| **Alcabre** | | | | | | | | | | | | |
| Mussel | – | – | – | – | 12.5 | 0 | 0 | 0 | 0 | 0 | 25 | 0 |
| Water | – | – | 0 | 0 | 75 | 0 | 10 | 0 | 40 | 0 | 100 | 0 |
| Sediment | 0 | 0 | 0 | 0 | 28.5 | 0 | 50 | 0 | 60 | 0 | 20 | 0 |
| Plankton | 0 | 0 | 0 | 0 | 88.8 | 0 | 0 | 0 | 20 | 0 | 80 | 0 |
| **Sor River** | | | | | | | | | | | | |
| Mussel | 25 | 25 | – | – | – | – | 100 | 0 | – | – | 25 | 0 |
| Oyster | 0 | 0 | 0 | 12.5 | – | – | 0 | 0 | – | – | 100 | 0 |
| Water | 0 | 0 | 0 | 0 | 40 | 0 | 25 | 0 | 100 | 0 | 60 | 0 |
| Sediment | 0 | 0 | 0 | 0 | 60 | 0 | 25 | 0 | 80 | 0 | 60 | 0 |
| Plankton | 0 | 10 | 0 | 0 | 80 | 0 | 0 | 0 | 0 | 0 | 100 | 0 |

identified bacterium in the total bacterial population of Alcabre and the Sor River is presented as a percentage in Fig. 5A. The percentages for each compartment (water, sediment, plankton, mussel or oyster) are illustrated in Fig. 5B,C. *V. harveyi* was the most abundant bacterium in Alcabre and the Sor River (45 and 52%, respectively). Up to 15% of the isolated bacteria from both locations were identified as *Vibrio* sp., with species undetermined. *V. fortis* was abundant in Alcabre (8%), and *V. diabolicus* and *V. anguillarum* were present in the Sor River (5 and 4%, respectively), but other *Vibrio* species, such as *V. tapetis*, *V. alginolyticus*, *V. campbellii*, *V. noatunensis* and *V. sinaloensis*, were also detected. Notably, species other than *Vibrio* were also identified (*Francisella* sp., *Agarivorans* sp., *Massilia* sp. and *Shewanella* sp.). Interestingly, 16% of the isolated bacteria in Alcabre and the Sor River were unidentifiable by sequencing (no homology with any sequence available in GenBank; Fig. 5A). *V. harveyi* and *Vibrio* spp. were detected in all of the samples, but few bacteria were observed in the water and

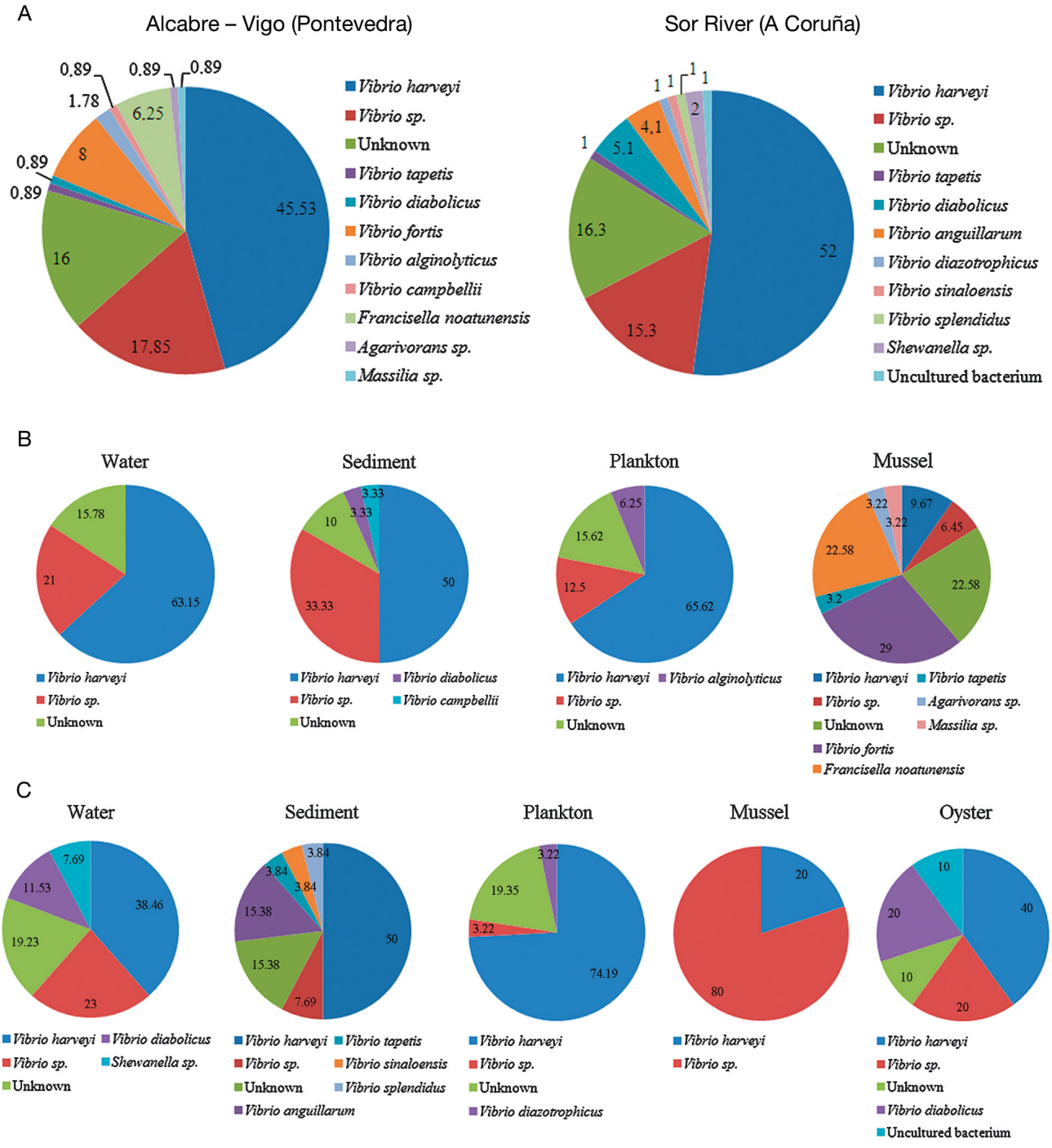


Fig. 5. Proportional representation of the Alcabre and Sor River bacteria identified by 16S rDNA sequencing. (A) Representation (%) of each group in the entire population of analysed bacteria from Alcabre and the Sor River. (B,C) Distribution of bacteria (%) within the different ecological compartments (water, sediment, plankton, mussel and oyster) in (B) Alcabre and (C) the Sor River

plankton (Fig. 5B). The highest diversity of *Vibrio* species was observed in the sediment; 6 different *Vibrio* species were isolated from sediment from the Sor River. Animals exhibited significant diversity in the number of isolated bacterial species. Four different *Vibrio* spp. and 3 types of bacteria unrelated to *Vibrio* were identified in mussels from Alcabre (Fig. 5B). In contrast, only 2 *Vibrio* groups (*V. harveyi* and

*Vibrio* sp.) were detected in mussels from the Sor River. At this location, oysters had greater bacterial diversity than mussels (Fig. 5C).

### Development of experimental disease models

Mussels were very resistant to the bacterial isolates used in this work, and high bacterial doses were needed to induce any mortality. Mortality after bath infection at 15°C was less than 5%, and no differences were observed between animals stressed by hypoxia and those that were continuously immersed (Fig. 6A, upper panels). However, when experimental infections were performed at 25°C, emersion increased mortality to 80 or 30% after a challenge with strains of *Vibrio splendidus* or *V. aestuarianus*, respectively (Fig. 6A, lower panels).

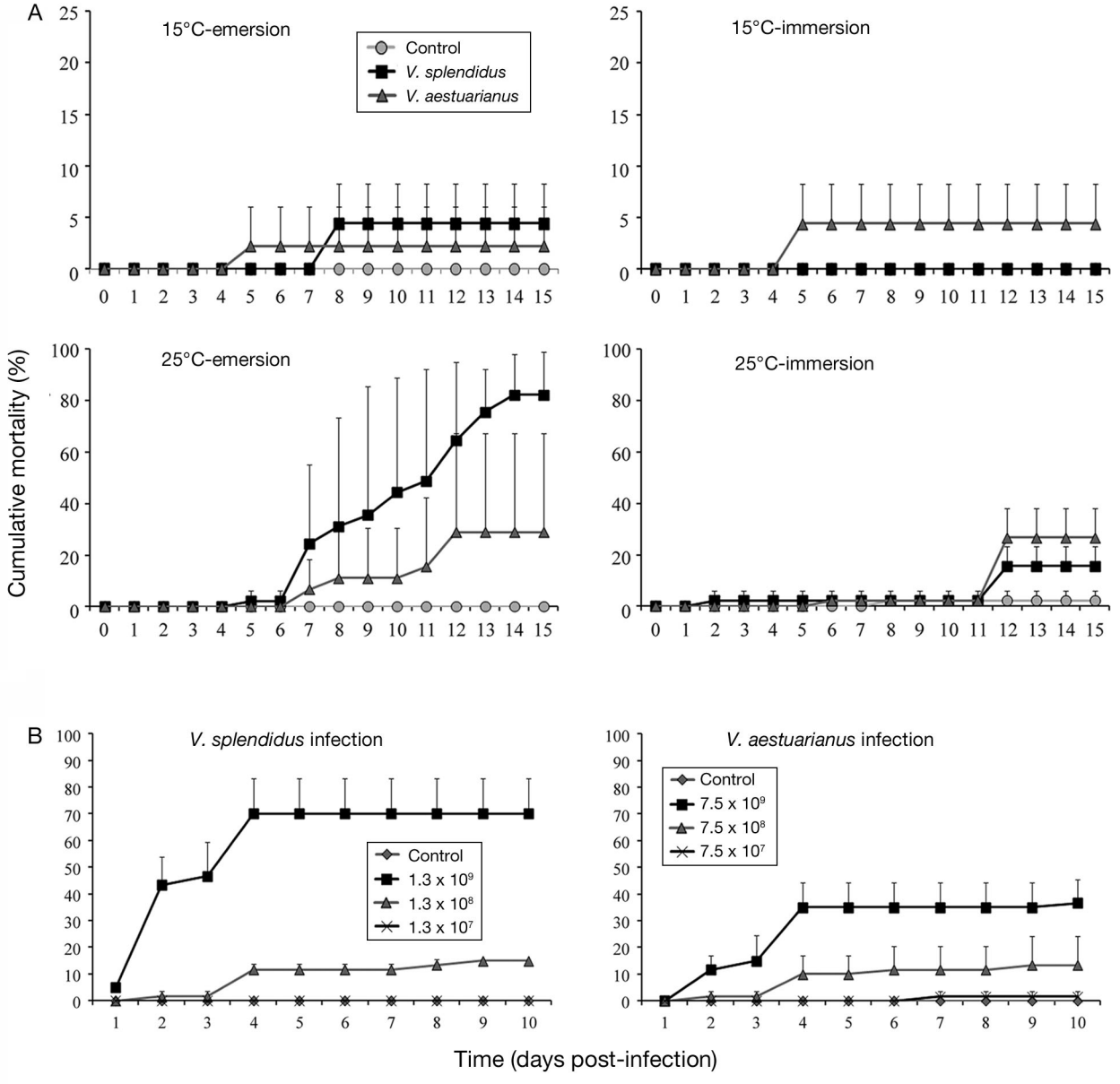


Fig. 6. (A) Cumulative mortality of mussels after a bath infection with *Vibrio splendidus* and *V. aestuarianus* at 15°C (upper panels) and 25°C (lower panels). The effect of an 8 h emersion, which mimics hypoxia-induced stress, on the cumulative mortality was determined (left panels; n = 540 animals). (B) Cumulative mortality of mussels after an intramuscular injection of 3 different doses of *V. splendidus* and *V. aestuarianus* at 15°C (n = 420 animals)

Intramuscular injection resulted in higher mortalities than bath exposure. Injecting the highest dose of *Vibrio splendidus* or *V. aestuarianus* resulted in 70 or 36.67% mortality, respectively, at the end of the experiment. A 10-fold dilution of the inocula significantly reduced mortality, and the lowest bacterial doses resulted in a mortality rate of less than 1.67% (Fig. 6B).

### Experimental infections of mussels with field *Vibrio* isolates

The isolates of *V. splendidus* from Alcabre were obtained from mussels collected in December (A5-M7) and April (A11M3B1 and A11M3B2). Strains of *V. splendidus* from the Sor River were isolated from mussels (S2M1, S8M5M1 and S12M3B2) collected in June, December and April and from oysters (S12-O2B1) obtained in April. The Sor River *V. aestuarianus* strains were isolated from mussels and plankton in August (S2M4 and S2P9, respectively) and from oysters in December (S4O8).

The cumulative mortality differed in mussels infected with the 7 isolates of *Vibrio splendidus*. Two out of 3 isolates obtained from Alcabre (A11M3B1 and A11M3B2) induced mortality similar to the reference strain (LGP32). Up to 70% of the animals infected with those isolates died at the end of the experiment. In contrast, animals infected with the A5M7 isolate exhibited 50% cumulative mortality (Fig. 7A). Interestingly, all of the *V. splendidus* isolates obtained from the Sor River resulted in fewer mortalities than the reference strain. Mortalities following injection of the 4 isolates ranged from 50 to 60% (Fig. 7B). Infection with the *V. aestuarianus* isolates resulted in variable cumulative mortality. Injection of the reference strain (01/032) resulted in 63% mortality, while injection of S4O8 resulted in 30% mortality. In contrast, infections of the mussel and plankton isolates produced similar, or even increased, mortality compared to the reference strain (64 and 89%, respectively; Fig. 7C).

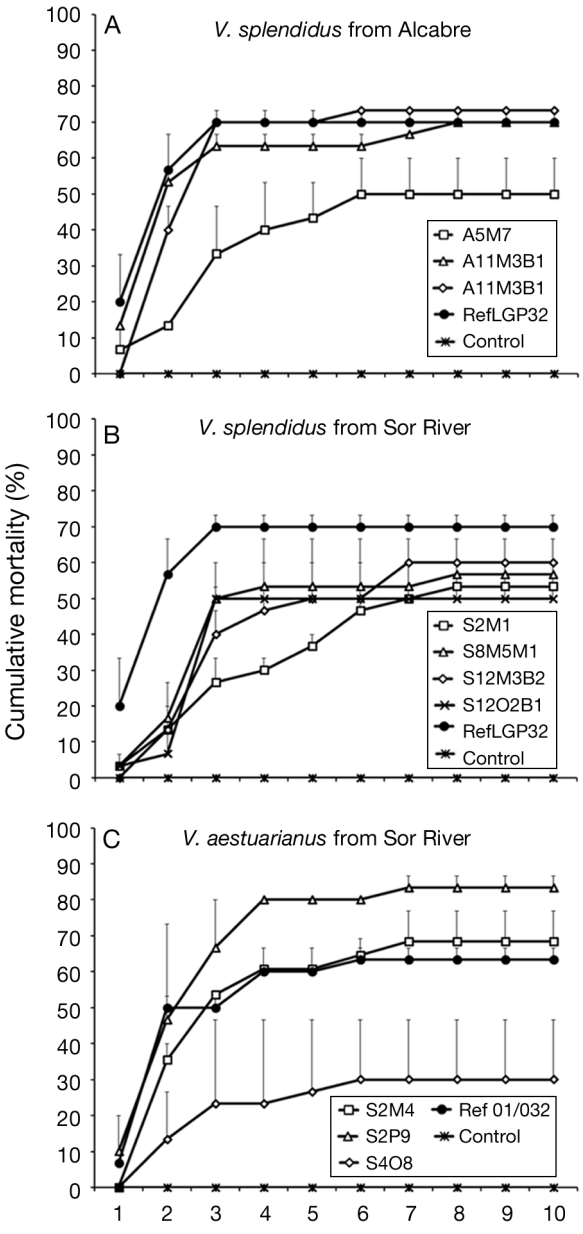


Fig. 7. Cumulative mortality from experimental infections by intramuscular injection of *Vibrio splendidus* isolated from (A) Alcabre and (B) the Sor River and (C) *V. aestuarianus* isolated from the Sor River. Data are means + SEM of 2 independent experimental infections (n = 390 animals)

## DISCUSSION

We selected 2 different regions of Galicia (NW Spain) based on their environmental and ecological characteristics and their actual production of bivalves. Alcabre is a marine ecosystem highly influenced by anthropogenic (industrial and recreational) activities and is one of the most important areas for mussel production in Spain (Caballero Miguez et al. 2009). The Sor River is a brackish water ecosystem with little human influence where natural mussel populations coexist with a population of Pacific oysters *Crassostrea gigas* introduced by experiments in the 1980s (Guerra et al. 1987, Iglesias et al. 2005).

The seasonal evolution of the environmental parameters, as well as the total bacterial concentrations,

reflected the ecological differences between the 2 sampling locations. In Alcabre, the moderate changes in water temperature and salinity agreed with previous data (Alvarez et al. 2005, Sousa et al. 2011). Based on the Pearson's R correlation results, the seasonal variations in salinity determined the abundance of culturable bacteria in the ecosystem. An increase in the total concentrations of bacteria in water and sediment was observed during the summer when the water temperature increased, as observed previously (Zdanowski & Figueiras 1997). In contrast, the increase in the bacterial load in the mussels between February and April was dependent on environmental parameters but was also influenced by upwelling events that enhanced primary production and the bacterial concentration in plankton (Hanson et al. 1986, Zdanowski & Figueiras 1999, Figueiras et al. 2006). In the Sor River, the extremely low salinity and temperature levels may be the primary factors governing the temporal and spatial distribution of bacteria in water and plankton. Salinity is recognised as one of the chief barriers to microbial communities (Bouvier & del Giorgio 2002, Trousselier et al. 2002, Crump et al. 2004).

*Vibrio* populations are strongly influenced by environmental conditions, including temperature, salinity, pH and nutrient availability (Lee et al. 2001, Armada et al. 2003, Thompson et al. 2004, Eiler et al. 2006, Martinez-Urtaza et al. 2008, Turner et al. 2009). Although no natural deaths were observed in the natural bivalve populations during this study, the environmental conditions in the 2 sampling locations were suitable for the growth of *Vibrio* spp. *V. splendidus* requires higher salinity than what is typically required by *V. aestuarianus* and other estuarine species, such as *V. parahaemolyticus* or *V. fluvialis*, for optimal growth (Thompson et al. 2004). However, we did not observe a strict correlation between seasonal changes in salinity and the concentration of both *Vibrio* species in the animals, as has been previously reported based on the use of classical culture-dependent techniques (Pujalte et al. 1999, Thompson et al. 2004). This may be because qPCR enables the detection of non-culturable and even non-viable bacteria. Moreover, it appears that the seasonal evolution of both *Vibrio* species in animals was more affected by internal factors than by environmental conditions. For example, similar seasonal patterns were observed in the mussels from the 2 sampling locations (different environmental conditions), but these patterns were distinct from those observed in oysters.

*Vibrio splendidus* and *V. aestuarianus* levels in mussels showed an inverse relationship, with the former most prevalent during the warm months and the latter dominating throughout the cold season, regardless of location. This temporal distribution has been reported for other *Vibrio* species (Parveen et al. 2008, Degremont 2011). An alternating pattern of dominance between 2 *Vibrio* species has been described in oysters (Degremont 2011) and suggests that the presence of one type of bacteria reduces that of the other. A similar mechanism of bacterial–bacterial antagonism has been reported to contribute to the regulation of *V. cholerae* in the marine environment (Long et al. 2005). Although the 2 species coexisted simultaneously in the same animal, isolates of *V. splendidus* were detected in all organs, whereas *V. aestuarianus* was essentially detected just in the mantle, suggesting differing tissue affinity. The reason for the alternating abundance of *V. splendidus* and *V. aestuarianus* in mussels remains unclear.

Water, sediment and plankton, as well as crustaceans and fish, can be reservoirs for *Vibrio* communities (Thompson et al. 2004, Turner et al. 2009). Although different *Vibrio* spp. were present in all of the environmental compartments, the sediment appeared to be the most important natural reservoir for these types of bacteria because it contained the greatest diversity of *Vibrio* species. Macro-fauna associated with natural mussel populations could also be reservoirs for *V. splendidus* because this bacterium was detected in cnidarians, molluscs and crustaceans, similar to what has been previously reported for other *Vibrio* species (Carman & Dobbs 1997).

Our culture-independent and culture-dependent results suggested that *Vibrio splendidus* is more prevalent than *V. aestuarianus* in the environment and animals, as described previously (Montilla et al. 1994, Arias et al. 1999, Pujalte et al. 1999, Beaz-Hidalgo et al. 2008, 2010). *V. aestuarianus* needed a previous enrichment to be detected compared with *V. splendidus*, since it was not detected by qPCR using DNA directly extracted from samples. Only after culture on TCBS and enrichment in peptone water could this bacterial strain be detected.

The variability of *Vibrio* communities in the environment and animal tissues was analysed after partial sequencing of the isolates that were unidentifiable by PCR. To date, the identification of *Vibrio* spp. isolated from aquaculture systems has been imprecise, and the taxonomy of *Vibrio* remains under revision (Vandenberghe et al. 2003, Beaz-Hidalgo et al. 2008, Noguerola & Blanch 2008). *V. harveyi*, which can be a primary pathogen in marine vertebrates and invertebrates (Austin & Zhang 2006), was the most prevalent clade, representing up to 52% of the total

sequenced bacteria, in accordance with previous publications (Arias et al. 1999, Castro et al. 2002, Alves et al. 2010). This bacterium was present in the plankton, water and sediment as previously described (Beaz-Hidalgo et al. 2010). *V. campbellii*, 1 of 4 species belonging to the *harveyi* clade (Cano-Gomez et al. 2011), also causes disease in reared aquatic organisms (Gomez-Gil et al. 2004) and was detected in sediment samples from Alcabre. The second most abundant group was composed of unidentified *Vibrio* spp., supporting the idea that the 16S rRNA gene is useful for grouping *Vibrio* species but has insufficient resolution to discriminate species (Cano-Gomez et al. 2011). As expected, the composition of the *Vibrio* communities in Alcabre and the Sor River differed. Our results agree with the established hypothesis that *Vibrio* population diversity is complex and is controlled by fluctuations in physiochemical parameters and by the abundance and composition of *Vibrio* reservoirs (Maugeri et al. 2004, Thompson et al. 2004, Turner et al. 2009, Gregoracci et al. 2012). Other *Vibrio* species associated with bivalve cultures, including *V. anguillarum*, *V. tapetis*, *V. diabolicus*, *V. fortis, V. noatunensis* and *V. sinaloensis* (Castro et al. 2002, Beaz-Hidalgo et al. 2010), have been detected in natural mussel populations.

Intramuscular injections revealed that *Vibrio splendidus* and *V. aestuarianus* were only moderately pathogenic in mussels compared with previous data on oysters. Pacific oysters appear to be more susceptible to the isolates of *V. aestuarianus* than to the *V. splendidus* isolates tested, as lower bacterial doses resulted in comparable mortality (Gay et al. 2004b, Garnier et al. 2007). This finding reflects the different susceptibilities of mussels and oysters to the same *Vibrio* isolates, similar to that previously described for clams (*Ruditapes philippinarum*) and oysters (*Crassostrea gigas*) (LeRoux et al. 2002).

Field studies have determined that bivalve mortality results from the interaction of environmental conditions, physiological status and the genetics of the host and the infectious agent, which could be an opportunist rather than a primary pathogen (Samain & McCombie 2008, Aranguren et al. 2012). In our bath infection experiments, temperature changes and hypoxic stress were included to mimic environmental changes because they have been implicated in bivalve deaths (Soletchnik et al. 1999, Lee et al. 2001). In this disease model, deaths only occurred when animals were infected with a high concentration of bacteria in adverse environmental conditions. This result reflects the extreme resistance of mussels to the bacterial pathogens tested.

Although the pathogenicity of the *Vibrio* strains isolated from wild mussel populations and natural reservoirs appeared to be low (high bacterial concentrations were needed to induce death in experimental infections), the fact that some strains induced more death than the reference strains highlights the potential risk of *Vibrio* spp. residing in plankton to bivalve populations. The differences in the mortality rates between different isolates of *V. splendidus* were expected since, as occurs in other bacterial species, different strains could present different degrees of virulence. Moreover, *V. splendidus* constitutes a clade containing numerous isolates of which only a few have been described (Thompson et al. 2004). Strains of *V. splendidus* isolated from the same animal after 2 different mortality events are never the same (Le Roux et al. 2002, Waechter et al. 2002). Moreover, several *Vibrio* species are generally isolated from the same individual, and a complex of cooperative interactions emerged between them during the pathogenic process in the molluscan host (Gay et al. 2004b). In contrast, isolates of *V. aestuarianus* can be considered as belonging to only 1 species, and only 1 strain could be isolated in the same animal.

In summary, our results showed that changes in environmental conditions significantly alter *Vibrio* communities throughout the seasons. The pathogenicity of *Vibrio* strains in cultured and natural bivalve populations depends not only on environmental variations but also on the physical condition of the animals. The presence of potentially pathogenic *Vibrio* species in natural mussel populations and in natural reservoirs confirms the importance of these types of studies in estimating the potential risk of these bacterial communities to the aquaculture sector. This work improves the actual limited knowledge about the occurrence of vibrios associated with mussel culture. Our results could aid in the design of preventive and control programmes to avoid the spread of the diseases, protecting the bivalve aquaculture industry.

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