Experimental infection of Mytilus edulis by two Vibrio splendidus -related strains: Determination of pathogenicity level of strains and influence of the origin and annual cycle of mussels on their sensitivity

Maud Charles, Suzanne Trancart, Elise Oden, Maryline Houssin

Experimental infection of *Mytilus edulis* by two *Vibrio splendidus*-related strains: determination of pathogenicity level of strains and influence of the origin and annual cycle of mussels on their sensitivity

Maud Charles[1, 2,*], Suzanne Trancart[2], Elise Oden[2], Maryline Houssin[1,2]

[1] Normandie Université, Université de Caen Normandie, UMR BOREA, CNRS-7208, IRD-207, MNHN, UPMC, UCN, Esplanade de la Paix, 14032 Caen Cedex 4, France

[2] LABÉO Frank Duncombe, 1 Route de Rosel, 14053 Caen Cedex 4, France

[*]Corresponding author: Maud Charles, LABÉO Frank Duncombe, Pôle Recherche, 1 Route de Rosel, 14053 Caen Cedex 4, France.
E-mail: maud.charles@outlook.fr

Abstract

This study aimed at assessing the pathogenicity of two *Vibrio splendidus*-related species and evaluating the influence of the origin and annual life cycle of mussels on their sensitivity during a bacterial challenge. Thus, *in vivo* infection assays were made with *Vibrio crassostreae* 7T4_12 and *Vibrio splendidus* 3G1_6, over respectively thirteen and nine months, on adult blue mussels from five recruitment areas in France. Two bacterial concentrations were tested: one consistent with the loads of *Vibrio* spp. in environment and mussel tissues (~ 10^5 CFU mL^{-1}) and another one much higher (~ 10^8 CFU mL^{-1}). The tested environmental concentration has no pathogenic effect whatever the time of year, the strain used and the origin of mussels. However, at the highest concentration, a pathogenic effect was observed only at specific moments, and one of the origins appeared to be more resistant. The physiological state of mussels –depending on the time of year– seemed significant in mussels' sensitivity, as their recruitment origin. This study is the first to test the pathogenicity of *V. splendidus*-related strains at concentrations close to what is found in the wild, over the annual cycle of mussels, and considering their origin.

Keywords:

mussels, *Vibrio splendidus*, pathogenicity, annual cycle, sensitivity, recruitment origin

1. Introduction

The marine mussels, *Mytilus* sp., are distributed worldwide (Gaitán-Espitia *et al.*, 2016) and constitute food source for aquatic and terrestrial animals including human beings, making them a high economic value product (FAO, 2016). The blue mussel industry occupies an important place in Europe, which is one of the main producers of mussels in the world with almost 600,000 tons per year. France is the second major European producer after Spain, with around 80,000 tons per year, most of them from aquaculture (FAO, 2018). Mussel farming in France is undertaken along the English Channel, the Atlantic, and the Mediterranean coasts and all mussel juveniles are collected –at spring for *Mytilus edulis*– in the wild. Thus, production is exposed to several external biotic and abiotic risks.

Indeed, filter feeding bivalves such as mussels are continually exposed to microbiota from the surrounding seawater and therefore vulnerable to attack from potential pathogens. Up to a recent time, adult mussels were considered to be very resistant to bacterial infections (Eggermont *et al.*, 2014) as well as to disease because of a well-working immune system (Venier *et al.*, 2011; Philipp *et al.*, 2012). *Mytilus edulis* was classified as a resilient cultivated species by mussel farmers insofar as they have only experienced very rare episodes of abnormal mortalities contrarily to what happened to the Pacific oysters, *Crassostrea gigas* (Barbosa-Solomieu *et al.*, 2015). In addition, these rare mass mortality events have not been attributed to pathogenic bacteria, but only to unfavourable environmental conditions or toxic algal blooms (Tsuchiya, 1983; Peperzak & Poelman, 2008). Nevertheless, since 2014, several important production areas on the French west coast have been affected by high and unusual mass mortality, resulting in significant losses and considerable economic consequences (Béchemin *et al.*, 2014; Allain & Bernard, 2016; FAO, 2016; Polsenaere *et al.*, 2017).

As the pathogenic bacteria *Vibrio splendidus* has been found in large abundance in French moribund mussels, its involvement was rapidly suggested (Béchemin *et al.*, 2015;

François *et al.*, 2015; Eggermont *et al.*, 2017). *Vibrio* spp. are indigenous, saprophytic and very abundant in aquatic environments such as estuaries, coastal waters, marine sediments and aquaculture environments all over the world (Colwell *et al.*, 1977; Ortigosa *et al.*, 1989). Some of these gram-negative bacteria are pathogenic to humans and other animals, including molluscs (Barbieri *et al.*, 1999; Romero *et al.*, 2014). Species belonging to the genus *Vibrio* are the most frequently isolated bacteria from molluscs and *V. splendidus* is widely distributed in marine ecosystems (Macián *et al.*, 2000); they are known to have impacted marine shellfish for many years with infectious diseases and have caused mortalities reported in the history of shellfish aquaculture (Beaz-Hidalgo *et al.*, 2010; Lemire *et al.*, 2015; Travers *et al.*, 2015). However, knowledge on their ecology and pathogenicity is still scarce.

In France, past mortalities of mussels greatly varied from one year to another, from one season to another and from one site to another (Lupo *et al.*, 2019). However, the French cultural practices, of natural larval recruitment make spat transfers to several growing sites on the Atlantic coast and English Channel, very common; the various growing areas in northern France are almost all supplied with spat from the same locations –roughly between the Loire and Garonne estuaries. Besides, even though mortality events have been reported at different times of the year, the majority of them took place at the end of winter and early spring, when the temperature increased (Soletchnik & Robert, 2016; Travers *et al.*, 2016; Cochennec-Laureau *et al.*, 2018; Glize & Gourmelen, 2018). It appeared that the etiology of these mortalities is complex; several factors could be involved like environmental conditions, infectious agent, physiology and genetic of the host, as suggested by Goulletquer *et al.* (1998) for mortality observed with *C. gigas.*

The objective of the present work was precisely to assess the influence of some internal and external factors of the host during a bacterial injection challenge. In this way, *in vivo* infection assays were made over a year, with adult blue mussels (*M. edulis*) from five

different recruitment areas, by using two *V. splendidus*-related species described as virulent for mussels from Brittany at high concentrations (Le Roux, pers. comm., unpubl. data): *Vibrio crassostreae* 7T4_12 and *V. splendidus* 3G1_6. The aims of this study were to evaluate (i) the pathogenicity and the virulence of these two strains on mussels by testing two different concentrations: one consistent with the general environmental loads of *Vibrio* spp. in seawater ~ $10^4/10^5$ CFU mL^{-1} (Pfeffer *et al.*, 2003; Eiler *et al.*, 2007), also corresponding to the bacterial concentrations that can be reached in *M. edulis* (equal to 2.9 x 10^4 CFU per mL or g according to Garnier *et al.*, 2007), and another one much higher ~ 10^8 CFU mL^{-1}; (ii) the influence of the first farming method used and original spat recruitment area on the sensitivity to *vibrio*sis; (iii) the role of physiological and morphological conditions of mussels –impacted by the season– in susceptibility to bacterial infections.

2. Materials and Methods

2.1 Bacterial strains and inoculum preparation

The two *V. splendidus*-related strains used for experimental infection were provided from the French institute, Ifremer. As part of their study (ANR 13-ADAP-0007-01 "OPOPOP"), they isolated many strains from a *Vibrio* population assembly isolated from oyster tissues (*C. gigas*) during and outside mortality events that hit oysters and mussels in the bay of Brest (Finistère, France) in 2014. Among the hundred species isolated, several appeared to be highly pathogenic for oysters (Bruto *et al.*, 2017) and mussels from Brittany (Le Roux, pers. comm., unpubl. data) of which *V. crassostreae* 7T4_12 and *V. splendidus* 3G1_6, that are used in this study, and respectively named S1 and S2 in the rest of the article. Twenty-four hours before infection, the bacteria were grown in fresh liquid sterile marine Zobell at 22 °C. The bacterial inocula were prepared using the fresh bacterial suspensions by diluting it in Artificial Sterilised Sea Water (ASSW) from 1:10 to 1:10,000,000 (serial ten-fold dilutions from 10^{-1} to 10^{-7}). The two dilutions used as inocula for experimental infections were 1:10

(10^{-1}) and 1:10,000 (10^{-4}). Bacterial load of pure bacterial suspension (BL) was defined by enumeration of Colony Forming Units (CFU) according to the standard NF ISO7218:2007 (AFNOR, 2007): BL (CFU mL^{-1}) = $\frac{\sum C}{V \times 1,1 \times d}$; where Σc is the sum of the colonies counted on the two plates of marine Zobell agar spread with two successive dilutions (10^{-6} and 10^{-7}) that incubated for 48 h at 22 °C; *V* is the volume that has been spread on plate (0.1 mL) and *d* is the dilution corresponding to the first dilution retained (10^{-6}).

2.2 Experimental mussels

Adult mussels used for this experiment were placed on intertidal mussel stakes ('*bouchot*') in the shellfish growing area off the coast of Bricqueville-sur-Mer, Normandy, France (48°55'05.8"N 1°34'23.9"W) since automn 2015. Spats were initially collected in early spring 2015 in five different recruitment areas: the cove of Port-Mânes (Ile Dumet, Loire-Atlantique, France); the Loire Estuary (La-Plaine-sur-Mer, Loire-Atlantique, France); the bay of Bourgneuf (Noirmoutier, Vendée, France); the bay of Aiguillon (Charron, Charente-Maritime, France); the Antioche Sound (Fouras, Charente-Maritime, France) (Fig. 1). These five origin groups are respectively named ID, LP, No, Ch, Fo in the rest of the article. The mussels from ID were different from the others: first, larval recruitment and first months of farming were made on longlines (while the others were on *'bouchot'*). Second, they were '*pelisse*' mussels, *i.e.* mussels from the upper layer of the collection rope that grew faster than the others. To leave space for the lower layer mussels, and to prevent these bundles of mussels from coming off, farmer collects and puts them back into 2.5 m long mesh tubes. Like initial collection rope, these nets are then coiled around a wooden pole at the growing area.

2.3 Experimental procedure of bacterial challenges

Experimental infections were performed monthly during one year for S1 (from April 2017 to April 2018) and during nine months for S2 (from August 2017 to April 2018). Mussels were

collected during low tide, transported in cooler bags to the laboratory and once there, immediately put back in tanks filled with oxygenated natural seawater at 15 °C overnight. The next day, they were anesthetised by bathing in a solution containing 40 g L^{-1} of magnesium chloride during 30 min. Then, 100 µL of inocula or ASSW (for negative controls) per mussel were injected into the posterior adductor muscle. In the rest of the article, the bacterial loads corresponding to the 100 µL of dilutions 10^{-1} and 10^{-4} have been respectively named C1 and C2. Following the injections, 20 mussels of each group (origin/strain/loads) were placed in separated tanks filled with 1 L of aerated seawater at 15 °C (Fig. 2). Thus, 100 mussels were injected for each condition (S1_C1; S1_C2; S2_C1; S2_C2 or negative controls). Water exchange and tank cleaning were carried daily for seven days and then, mortalities were recorded. All mussels that did not spontaneously close their valves when the seawater was removed were considered as dead. Dead mussels were sampled for biometry (flesh weight and shell length) and microbiological analysis. The objective was to verify whether among the main bacteria found in the tissues of dead mussels, the strain initially injected was present (Koch's fourth postulate).

2.4 Microbiological and molecular analyses of dead mussels

Tissues of dead mussels were mashed with scalpels and a volume of 450 µL of ASSW was added to 50 mg of the homogenate. After stirring and a pulse centrifugation to pellet cellular debris, ten-fold dilutions of supernatant were made and 100 µL of 1: 10,000 (10^{-4}) and 1: 100,000 (10^{-5}) dilutions were sown on Zobell marine agar and incubated for 48 h at 22 °C. For each plate, the three predominant bacterial colonies were re-isolated in order to ensure their purity. Then, DNA extraction of these three predominant colonies was performed by heating the colony placed in 250 µL of purified water (DNA/nuclease free water) for 10 min at 95 °C. The successful extraction was confirmed by DNA quantification with NanoDrop™ 2000c spectrophotometer (ThermoFisher Scientific™, Waltham, MA USA) and strains were

then characterised by molecular analysis. One TaqMan® real-time PCR targeting the *16S rRNA* gene of *V. splendidus*-related strains (PCR1) (Oden *et al.*, 2016) was carried out on a Smart Cycler® (Cepheid, USA); the primers used for PCR1 were *SpF1* 5'ATCATGGCTCAGATTGAACG3' and *SpR1* 5'CAATGGTTATCCCCCACATC3' (Nasfi *et al.*, 2015) and the probe *SpProbe* (5'-3') FAM-CCCATTAACGCACCCGAAGGATTG-BHQ1. The reaction volume of 25 µL contained 12.5 µL of Premix Ex Taq® 2 X Takara® (Lonza, Verviers, Belgium), 0.5 µL of each primer (20 µM), 0.5 µL of probe (10 µM), 9 µL of purified water and 2 µL of extracted DNA (replaced with 2 µL of purified water in the negative control). The thermal cycling profile was 95 °C for 10 s, followed by 40 cycles at 95 °C for 5 s and 62 °C for 30 s. Then, when PCR1 was positive, the objective was to differentiate strains of *V. splendidus*-related species from each other and identify the inoculated strain.

2.5 Genotyping of V. splendidus-related strains

A conventional PCR targeting the housekeeping genes *mreB* of *V. splendidus*-related strains (PCR2) was performed since it is the most discriminant gene for the identification of closely related strains among the *Splendidus* clade (Oden *et al.*, 2016); the primer pair for PCR2 was *mreB*-F 5'CTGGTGCTCGYGAGGTTTAC3' and *mreB*-R 5'CCRTTYTCTGAKATATCAGAAGC3′. For the conventional PCR, typical 25 µL reaction mixtures contained 12.5 µL of Premix Ex Taq® 2 X Takara® (Lonza, Verviers, Belgium), 0.5 µL of each primer (20 µM), 9.5 µL of purified water and 2 µL of DNA template (replaced with 2 µL of purified water in the negative control). Conventional PCR amplifications were carried out in a T100™ Thermal Cycler (Bio-Rad, France) and the thermal program was as follows: 10 s at 95 °C; 30 cycles for 10 s at 95 °C, 30 s at 55 °C, 40 s at 72 °C and a final extension of 3 min at 72 °C. PCR products were then analysed with QIAxcel® Advanced

System (Qiagen, Courtaboeuf, France) and sent to Eurofins MWG Operon (Ebersberg, Germany) to be purified and subsequently sequenced.

2.6 Phylogenetic analyses

The *mreB* sequences were aligned using a multiple sequence alignment Multiple Sequence Comparison by Log-Expectation (MUSCLE); phylogenetic analyses were performed in MEGA7 (Kumar *et al.*, 2016) using the Neighbor Joining method (Tamura *et al.*, 2013) and the maximum composite likelihood model with a bootstrap of 1000 replications. Thus, the sequences of the *mreB* gene of *V. spendidus*-related strains from dead mussels could be compared with the sequences of the injected strains and with forty-seven different reference bacterial strains (whose forty-two belonging to the *Splendidus* clade; see *2.1.* in Oden *et al.* 2016). *Vibrio aestuarianus* 02/041, *Vibrio ordalii* 12B09 and *Vibrio penaeicida* AQ115, were provided from Genomic of *Vibrio* Research Department (CNRS Roscoff, France) and used as *Splendidus* clade outsiders.

2.7 Establishment of periods related to the annual life cycle of mussels

In order to estimate the role of physiological conditions of mussels in susceptibility to bacterial infections, ‘periods’ have been defined, related to the mussel cycle. The cycle of *M. edulis* in cold waters has been known for several years (Boromthanarat *et al.* , 1987; Gosling, 2003): gametogenesis begins in late summer and early autumn (Sept./Oct.), gonads are ripe and animals mature in early winter (Dec./Jan.). The emission of gametes in water occurs in late winter and early spring (Feb.-Apr.) and the resting stage begins in late spring and early summer (June). Thus, the following periods have been defined: from February to April = period A; from May to July = period B; from August to October = period C; from November to January = period D.

2.8 Statistical analyses

Statistical analyses and graphical representations were performed by using R software, version 3.5.1 and Microsoft Excel software, version 2016. The differences in mortality observed between (i) the conditions tested (negative control, C1 and C2) and (ii) the periods of the year, were tested using a Kruskal-Wallis test (K-W). When significance was obtained, a pairwise Wilcoxon test was done. The difference in virulence between S1 and S2 was tested by comparing two observed distributions with a chi-squared test of independence. To finish, the numbers of dead mussels observed for each origin were compared with a chi-squared goodness-of-fit test. The statistical analyses described above were carried out on the number of dead mussels found positive for the strain initially injected (S1- or S2-positive); except for the first test (which compared dead mussel counts among all conditions), the comparison was first made between all the dead mussels, then only with the S-positive. Regarding biometry data, normality was confirmed with a Shapiro test and an ANOVA was done. When results were significant, a *post-hoc* test (Tukey test) was performed. Statistical significance was accepted for $*p < 0.05$ and $**p < 0.01$.

3. Results

3.1 Bacterial inocula concentrations

The BL of all experiments performed with S1 and S2 are reported in Table 1. Mussels were inoculated with average bacterial loads equal to 3.12×10^7 CFU (S1_C1) and 3.12×10^4 CFU (S1_C2) for S1, and 2.23×10^7 CFU (S2_C1) and 2.23×10^4 CFU (S2_C2) for S2.

3.2 Overall obtained mortalities and strain-positive mussels

The numbers of infected mussels, dead mussels and strain-positive dead mussels obtained for each experimental infection are reported in Table 1. On all experimental infections made with S1, 1,300 mussels were inoculated with S1_C1 and 189 died (mean = 15; min = 0; max = 65), and 1,300 mussels were infected with S1_C2 and 26 died (mean = 2; min = 0; max = 10). On

all bacterial challenges performed with S2, 900 mussels were inoculated with S2_C1 and 326 died (mean = 36; min = 0; max = 86), and 900 mussels were infected with S2_C2 and 19 died (mean = 2; min = 0; max = 11). On average over all experimentations, S1 was found in 48 % (90/189) of dead mussels in condition S1_C1 and in 4 % (1/26) in S1_C2, and S2 was found in 50 % (163/326) of dead mussels in condition S2_C1 and in 5 % (1/19) in S2_C2. None of the dead mussels in the negative controls (n = 15) were positive in S1 or S2. Figure 3 shows one of the phylogenetic analysis; in total, 255 *mreB* sequences were aligned over all these bacterial challenges.

3.2.1 Comparison of mortality by tested conditions (strains / loads)

The observed mortality between the tested conditions differed significantly (K-W, S1: p-value** = $2.7.10^{-5}$; S2: p-value** = $2.1.10^{-6}$). For S1 and S2, the mortality obtained at the highest concentration (C1) is significantly higher from the others (Wilcoxon test, S1: C1/C2 p-value* = 0.02 and C1/controls p-value** = 0.009; S2: C1/C2 p-value** = 0.007 and C1/controls p-value** = 0.0006). There was no difference between C2 and negative controls for both strains (positive to the strain or not).

3.2.2 Comparison of mortality by period

Figure 4 illustrates the obtained mortality with S1 (Fig. 4A) and S2 (Fig. 4B) at each month of each period at the highest concentration tested (C1). The obtained mortality differs according to the defined periods and therefore according to the life cycle of mussels (K-W, S1: p-value* = 0.04; S2: p-value* = 0.02). For S1, period A is significantly different from periods B, C and D (Wilcoxon test, p-values* < 0.05). For S2, there is no difference between period A and C (Wilcoxon test, p-value = 0.07) but significative difference exists between, A or C, and period D (Wilcoxon test, p-values* < 0.05). What is also quite remarkable in this figure is the percentage of S-pos dead mussels; over the period A, the percentage of S-pos dead mussels was on average < 50 % for S1 and S2 while for period C, it was ~ 75 % for S2.

3.2.3 Comparison of the pathogenicity of the two strains over the common time interval

By comparing the numbers of dead mussels following infection by S1 and S2 over the common time interval, it appears that S2 was globally significantly more pathogenic than S1 (chi-squared = 68.25 > chi-squared$_{0.01}$ = 15.08, df = 5; p-value** < 0.01). When the obtained mortalities for each month are compared, it is notable that the mortalities caused by S2 are significantly higher in most months (Fig. 5). Indeed, S2 caused mortality during period C whereas S1 did not; moreover, it caused mortality over the entire period A whereas S1 caused significantly less, with a bell-shaped evolution during the period and, with a peak in March. However, despite the difference in pathogenicity, S1 and S2 both cause similar mortalities in March (S-positive dead mussels).

3.2.4 Comparison of mortality depending on spat recruitment sites

Among all S1- or S2-positive dead mussels (n=253), 12 % were from ID (30/253), 19 % from LP (48/253), 22.5 % from Ch (57/253), 22.5 % from Fo (57/253) and 24 % from No (61/253) (Fig. 6). The number of dead mussels originating from ID is significantly lower than the others (chi-squared = 13.87 > chi-squared$_{0.01}$ = 13.27, df = 4; p-value** < 0.01).

3.3 Biometry

Figure 7 shows the mean values (± SEM) of length and flesh weight of dead mussels according to their origin. Several differences appear between the biometric values of the mussels, but the most notable is for the mussels of ID. Overall, they were much larger and fleshier than the others. On the contrary, the mussels from LP were slightly smaller and less fleshy.

3.4 Other observations

The parasite, *Mytilicola intestinalis*, was fortuitously found in the intestinal tract of some dead animals. Even though, the objective of the study was not to search for it, parasites easily visible to the naked eye (> 0.25 mm) were counted still; between one to five individuals were

observed in more than a third of dead mussels. Also, less than 10 % of the mussels had their mantle full of macroscopically visible trematode sporocysts or metacercaria.

For experiments done during period A, an interesting phenomenon was observed. Just a few hours after injection, the mussels that have been infected at the highest concentration with S1 and S2, began to lay their gametes in water tank (Fig. 8A and Fig. 8B). When spawning was observed, the water was immediately changed. However, this occurred several times during the first 2-3 days after infection. For dead mussels collected during this period, more than half had a mantle with some remnants of gametes not expelled (un-spawned gametes) (Fig. 8C and Fig. 8D) or totally empty (like the valve on the right in Fig. 8D).

4. Discussion

4.1 Pathogenicity of V. crassostreae 7T4_12 and V. splendidus 3G1_6

Performing an enumeration to obtain the BL of bacterial suspension of each inoculum allowed to verify precisely the tested concentrations. By achieving the objective of testing bacterial concentrations consistent with those found in seawater or in mussel tissues (~ 10^4 CFU animal^{-1}; Eiler *et al.*, 2007; Garnier *et al.*, 2007; Pfeffer *et al.*, 2003), as well as high concentrations (~ 10^7 CFU animal^{-1}), different results was observed. Most of the experimental infections performed on mussels (Parisi *et al.*, 2008; Tanguy *et al.*, 2013; Ben Cheikh *et al.*, 2016, 2017) or oysters (Gay *et al.*, 2004; Garnier *et al.*, 2007; Travers *et al.*, 2017) were made by injections with high bacterial strain concentrations around 10^7/10^8 CFU animal^{-1} which is quite far from those found in the field. On this point, Travers *et al.* (2017) mentioned that defining a strain as virulent through high concentration injections was a debatable issue. As for Goudenège *et al.* (2015), they determined three categories of strains as part of their high-throughput bacterial infections: virulent (> 50 % mortalities at 10^2 CFU animal^{-1}), non-virulent (< 50 % mortalities at 10^7 CFU animal^{-1}) and intermediate (*i.e.* pathogenic; an effect is observed only at 10^7 CFU animal^{-1}). They have thereby shown that a real virulent strain

induces high mortality rates on oysters, even at very low concentrations (10^2 CFU animal^{-1}). The present study has indicated that even by intramuscular injection of a pure bacterial solution of S1 or S2 at 10^4 CFU animal^{-1}, there was no significant mortality whatever the time of the year. Thus, it is possible to affirm that these two strains are not virulent. In contrast, it appeared that, with a high concentration (10^7 CFU animal^{-1}), both strains were capable to cause significant mortalities (14.5 % in period C for S2, and in period A, 19.5 % and 38.5 % respectively for S1 and S2). According to the classification of Goudenège *et al.* (2015), these two strains could be defined as, at most, non-virulent or pathogenic; because it entirely depends on the time of year and that is part of what this study highlights. Indeed, these two strains could have wrongly been defined as pathogenic or non-pathogenic, depending on the period during which bacterial challenges were performed. In any case, it appeared that S2 was overall more pathogenic than S1 at 10^7 CFU animal^{-1} because it caused significantly more mortality. Besides, S2 caused mortality at a time when S1 caused almost none (period C), and the mortalities observed during this period appear to be actually due to S2 insofar as it is found in three out of four mussels. Indeed, to verify the presence of the inoculated strain seems to be also an important detail to check. During period A –more particularly in March– similar mortalities were obtained with S1- and S2-pos mussels and the injected strains were absent in more than half of the total of dead mussels (over the period A, only 58 out of 122 mussels were S1-pos and only 115 out of 254 mussels were S2-pos); however, higher mortalities were observed with S2 during period A. It can be hypothesized that, in order to disrupt the animal at this vulnerable period, the injected bacteria did not necessarily need to get the upper hand, compared to other commensal bacteria, as during less sensitive periods such as period C. Therefore, if the presence of bacteria had not been investigated, and the experiments had only been carried out at that time of the year, the strains would have been wrongly defined as very pathogenic.

4.2 Influence of the annual life cycle during a bacterial challenge

In this study, the observation of forced egg laying following bacterial challenges suggests that the physiological condition of mussels plays a decisive role in the observed mortality. Carrying out the same bacterial challenges monthly over several months made it possible to study and compare mussel response to the same bacterial challenges, in the same laboratory conditions, and thus to evaluate the role of the physiological status of the host. These experimentations showed that there was a seasonality in mortality caused by the bacteria pointing out a link between sensitivity to infection and the annual cycle of mussels. The annual cycle of cold-water mussels includes several stages: growth, ripening of gametes, spawning, and gonad redevelopment; this cycle is adjusted according to environmental conditions (temperature and food supply mostly; Gosling, 2003). During summer, gonadal reserves are accumulated and once the minimum level is reached, the gametes development starts –usually in October–, it continues throughout the winter months and, in February, the gonads are ripe. At that point, mussels are ready to spawn waiting for natural exogenous factors (rise in water temperature, salinity change, etc.). Nevertheless, under laboratory conditions, spawn could be induced by a temperature shock or a physical stimulation –like scraping the shell or pulling the byssus threads (Gosling, 2003; Seed & Suchanek, 1992). This study showed that the inoculation of a high bacterial load constitutes a stress strong enough to induce spawning in mussels that are in an advanced state of maturation or ripe (period A) and no matter what strain used. Soletchnik *et al.* (1999) described this physiological condition as one of the major factors in the numerous mortality episodes observed in oysters and in mass mortality episodes of French mussels in 2014 and 2015 (Soletchnik & Robert, 2016). Spawning is an energy demanding process during which mussels are in poor condition with low glycogen content (depleted reserves) which makes them particularly vulnerable to stressful environmental conditions (Bayne *et al.*, 1980; Bayne & Worrall, 1980; Najdek &

Sapunar, 1987; Myrand *et al.*, 2000). The results observed here are consistence with these studies. Indeed, the high mortalities obtained during period A were the outcome of global weakness of mussels –that accompanies this triggering of spawning– and the inoculation of a high concentration of pathogenic bacteria. It is even more obvious because, as for S1, no mortality has been observed during other periods. Inoculation induced significant stress that triggered spawning; it is then possible to wonder: if the nature of the stress had been different (*e.g*. chemical contaminant spill or toxic algae bloom), would the spawning have been triggered and what would have been the result on the observed mortality rates? Nevertheless, in the case of S2, the strain seems to have increased mortality more strongly during this period than for S1, but as showed above, S2 caused mortality during period C while S1 did not. This is interesting because this post-spawning period corresponds to the sexual rest during which glycogen content is improved and is accumulated as an energetic reserves (Bayne & Worrall, 1980); it shows that mussels remain sensitive during this period and this is also what Myrand *et al.* (2000) observed in their analysis of the link between reproduction and summer mortality in Québec mussels. On the other hand, period D appears to be the interval when mussels are more robust since no significant mortality has occurred. During this period, energy reserves are usually used to meet the energy needs of gametogenesis and in December, mussels are full –mature– with gonads that occupy all the space, without being ready or able to spawn. The lack of observed mortality suggests that they have accumulated enough energy to fight against and deal with high infection. It seems that this time of the year is the most appropriate time to test a strain for virulence or pathogenicity; indeed, if strain causes mortality at that moment, then it will cause probably more at another time.

4.3 Mortality according to original spat recruitment site

Moving forward in the study, it became very clear that the mussels from ID were different from the others. They were larger, fleshier and died significantly less than the others

as a result of bacterial challenge. However, all the mussels were the same age and had the same living conditions over three quarters of their lifespan since they had been on '*bouchot'* during the same time (~ the last 18 months at the beginning of the study); only the recruitment site and the first five months of farming method varied. Despite these more than 18 months of identical life, the mussels from ID were significantly bigger and more robust. Thus, two hypotheses to explain their robustness can be proposed: it would either come from (i) the morphology of the mussels, influenced by the farming method used during the first months; or from (ii) the genetic inheritance of the mussels, influenced by the genetic characteristics of the natural local population; or perhaps from a combination of both factors.

Mussel growth is mainly influenced by food availability (Gosling, 1992) and the longlines farming method –with continuous immersion– allows mussels to have access to trophic resources without interruption. Mussels grown on longlines have higher growth performance than mussels grown on '*bouchot'* (Prou & Goulletquer, 2002); and this is what was observed for the ID mussels which were larger from the beginning to the end of the study. As described above, the life cycle of mussels is very energy-intensive, so larger individuals have logically more glycogen reserves, which probably allows them to have a negative energy balance later than others.

Several authors have compared the growth rates and the mortality of mussels by transferring samples from different natural populations in the same growing areas (Dickie *et al.*, 1984; Mallet *et al.*, 1986, 1987; Fuentes *et al.*, 1994). These studies have demonstrated that survival capacities of mussels –and so variations in mortality– could be attributed to different origins (source of mussel stocks). Mallet and Haley (1984) indicated that mortality variation from one origin to another evidences the differences between populations of *M. edulis*; this is also what Dickie *et al.* (1984) observed thanks to the significant differences in genetic constitution among the stock used in their experiments. Following this, Mallet *et al.* (1986) showed that

viability is highly heritable in *M. edulis* populations and, very recently, Dégremont *et al.* (2019) revealed that selected populations of French *M. edulis* were significantly more resistant to mortality outbreaks than natural unselected populations. *Mytilus edulis* and *M. galloprovincialis* are native species to the French coast, and numerous different natural populations exists and are capable to hybridise with each other. Thus, the Atlantic coasts of France is very complex –in terms of genetic structure– with several mosaic hybrid zone between *M. edulis* and *M. galloprovincialis* (Bierne *et al.*, 2003; Riquet *et al.*, 2017). It is therefore possible that some natural French populations could be more resistant than others.

Snieszko (1974) wrote 'an overt infectious disease occurs when a susceptible host is exposed to a virulent pathogen under proper environmental conditions' which means that three elements –pathogen, environment, and status of the host– strongly interact in the disease. In some cases, only two unfavourable elements seem sufficient to provoke mortality (Garnier *et al.*, 2007). This fact was observed in this study since the experiments were conducted under the same laboratory conditions. What could be added to Snieszo's quote is that within the host itself, many parameters –physiological state, morphology, and probably genetic inheritance– can influence the host's response to the disease.

Overall, the results provide information on the mortalities observed in mussels, *M. edulis*, over the annual life cycle, in response to bacterial stress. To our knowledge, this is the first study to test the pathogenicity of *V. splendidus*-related strains at concentrations close to what is found in the wild, over the entire annual cycle of mussels, and considering their origin. The results demonstrate that these two strains are not virulent for mussels and that the mortalities obtained during *in vivo* bacterial challenges are distinct depending on the physiological state of mussels –reliant on the time of the year–, and on the recruitment origin of the spat. Thus, to

test the virulence or the pathogenicity of a bacterial strain on mussels, it seems consistent to consider the origin of the population and the state of maturity of mussels and thereby, avoid bacterial challenges during the spawning period of the mussels because this is not strictly representative of the pathogenicity of the strain.

5. Acknowledgements

The authors thank Ifremer and Dr. F. Le Roux (Genomic of *Vibrio*-Station Biologique de Roscoff) for giving us *Vibrio* spp. strains, Patrick Céron for making of the map and Dr. D'Rego for the proofreading of English. The Regional Shellfish Committee of Normandy/North Sea (CRC Normandie/Mer du Nord) and the laboratory LABÉO financially supported this study. Maud Charles received co-funding from the Normandy region and from the laboratory LABÉO Frank Duncombe.

No conflict of interest declared.

The authors confirm that one part of the data supporting the findings of this study is available within the article and the other part is available on request from the corresponding author.

6. References

AFNOR (2007). *NF EN ISO 7218 : Microbiologie des aliments - Exigences générales et recommandations*.

Allain G & Bernard I (2016). *Les mortalités de moules en 2014 et 2015 vues par les professionnels. Compte-rendu de la phase 1 : synthèse sur l'émergence, la propagation et l'installation des mortalités*. Comité Régional de la Conchyliculture Bretagne Nord.

Barbieri E, Falzano L, Fiorentini C, Pianetti A, Baffone W, Fabbri A, Matarrese P, Casiere A, Katouli M, Kühn I, Möllby R, Bruscolini F & Donelli G (1999). Occurrence, Diversity, and Pathogenicity of Halophilic *Vibrio* spp. and Non-O1 *Vibrio cholerae* from Estuarine Waters along the Italian Adriatic Coast. *Appl Environ Microbiol* **65,** 2748–2753.

Barbosa-Solomieu V, Renault T & Travers M-A (2015). Mass mortality in bivalves and the intricate case of the Pacific oyster, *Crassostrea gigas*. *J Invertebr Pathol* **131,** 2–10. doi: 10.1016/j.jip.2015.07.011

Bayne B, Brown D, Harrisson F & Yevich P (1980). Mussel health. In *The International Mussel Watch*, US National Academy of Science., pp. 163–235. E. D. Goldberg, Washington DC.

Bayne B & Worrall C (1980). Growth and Production of Mussels *Mytilus edulis* from Two Populations. *Mar Ecol Prog Ser* **3,** 317–328. doi: 10.3354/meps003317

Beaz-Hidalgo R, Balboa S, Romalde JL & Figueras MJ (2010). Diversity and pathogenecity of *Vibrio* species in cultured bivalve molluscs. *Environ Microbiol Rep* **2,** 34–43. doi: 10.1111/j.1758-2229.2010.00135.x

Béchemin C et al. (2014). *Surmortalités de la moule bleue Mytilus edulis dans les Pertuis Charentais* (mars 2014). Ifremer. Available at: http://archimer.ifremer.fr/doc/00229/34022/32387.pdf [Accessed December 19, 2016].

Béchemin C, Soletchnik P, Polsenaere P, Le Moine O, Pernet F, Protat M, Fuhrmann M, Quéré C, Goulitquer S, Corporeau C & others (2015). Episodes de mortalité massive de moules bleues observés en 2014 dans les Pertuis charentais. *Bull Épidémiologie Santé Anim Aliment*, 6–9. Available at: http://archimer.ifremer.fr/doc/00312/42343/ [Accessed December 19, 2016].

Ben Cheikh Y, Travers M-A & Le Foll F (2017). Infection dynamics of a *V. splendidus* strain pathogenic to *Mytilus edulis* : *In vivo* and *in vitro* interactions with hemocytes. *Fish Shellfish Immunol* **70,** 515–523. doi: 10.1016/j.fsi.2017.09.047

Ben Cheikh Y, Travers M-A, Morga B, Godfrin Y, Rioult D & Le Foll F (2016). First evidence for a *Vibrio* strain pathogenic to *Mytilus edulis* altering hemocyte immune capacities. *Dev Comp Immunol* **57,** 107–119. doi: 10.1016/j.dci.2015.12.014

Bierne N, Borsa P, Daguin C, Jollivet D, Viard F, Bonhomme F & David P (2003). Introgression patterns in the mosaic hybrid zone between *Mytilus edulis* and *M. galloprovincialis*. *Mol Ecol* **12,** 447–461. doi: 10.1046/j.1365-294X.2003.01730.x

Boromthanarat S, Deslous-Paoli JM & Heral M (1987). Reproduction et recrutement de *Mytilus edulis* L. cultivée sur les bouchots du bassin de Marennes-Oléron. *Haliotis* **16,** 317–326. Available at: https://archimer.ifremer.fr/doc/1987/publication-2865.pdf [Accessed May 31, 2019].

Bruto M, James A, Petton B, Labreuche Y, Chenivesse S, Alunno-Bruscia M, Polz MF & Le Roux F (2017). *Vibrio crassostreae*, a benign oyster colonizer turned into a pathogen after plasmid acquisition. *ISME J* **11,** 1043–1052. doi: 10.1038/ismej.2016.162

Cochennec-Laureau N, Schapira M, Bouget J-F, Manach S, Retho M, Le Merrer Y, Schmitt A, Pierre-Duplessix O, Gabellec R, Normand J & Combette A (2018). *Expertise sur le contexte hydro-climatologique de la baie de Pénestin pour décrire les conditions physio-chimiques environnementales et l'impact possible sur les mortalités de moules.* Ifremer, DDTM 56, DML, SAMEL, Unité Cultures Marines.

Colwell RR, Kaper J & Joseph SW (1977). *Vibrio cholerae*, *Vibrio parahaemolyticus*, and Other *Vibrio*s: Occurrence and Distribution in Chesapeak Bay. *Science* **198,** 394–396. doi: 10.1126/science.910135

Dégremont L, Maurouard E, Rabiller M & Glize P (2019). Response to selection for increasing resistance to the spring mortality outbreaks in *Mytilus edulis* occurring in France since 2014. *Aquaculture* **511,** 734269. doi: 10.1016/j.aquaculture.2019.734269

Dickie LM, Boudreau PR & Freeman KR (1984). Influences of Stock and Site on Growth and Mortality in the Blue Mussel (*Mytilus edulis*). *Can J Fish Aquat Sci* **41,** 134–140. doi: 10.1139/f84-013

Eggermont M, Bossier P, Pande GSJ, Delahaut V, Rayhan AMd, Gupta N, Islam SS, Yumo E, Nevejan N, Sorgeloos P, Gomez-Gil B & Defoirdt T (2017). Isolation of *Vibrio*naceae from wild blue mussel (*Mytilus edulis*) adults and their impact on blue mussel larviculture. *FEMS Microbiol Ecol* **93,** 11. doi: 10.1093/femsec/fix039

Eggermont M, Tamanji A, Nevejan N, Bossier P, Sorgeloos P & Defoirdt T (2014). Stimulation of heterotrophic bacteria associated with wild-caught blue mussel (*Mytilus edulis*) adults results in mass mortality. *Aquaculture* **431,** 136–138. doi: 10.1016/j.aquaculture.2014.01.014

Eiler A, Gonzalez-Rey C, Allen S & Bertilsson S (2007). Growth response of *Vibrio cholerae* and other *Vibrio* spp. to cyanobacterial dissolved organic matter and temperature in brackish water. *FEMS Microbiol Ecol* **60,** 411–418. doi: 10.1111/j.1574-6941.2007.00303.x

FAO (2016). Demand for European mussels within the EU on the decline. In *GLOBEFISH - Information and analysis on world fish trade*. http://www.fao.org/in-action/globefish/market-reports/resource-detail/en/c/450826/. Available at: http://www.fao.org/in-action/globefish/market-reports/resource-detail/en/c/450826/. [Accessed May 21, 2019].

FAO (2018). Fisheries and aquaculture software. FishStatJ - software for fishery statistical time series. In *FAO Fisheries and Aquaculture Department*. http://www.fao.org/fishery/. Available at: http://www.fao.org/fishery/. [Accessed May 21, 2019].

François C, Garcia C, Lupo C, Travers MA, Morga B, Tourbiez D, Faury N, Haffner P, Serpin D, Dubreuil C, Chollet B, Baillon L, Lapègue S & Renault T (2015). *Bilan 2014 du réseau Repamo - Réseau national de surveillance de la santé des mollusques marins*. Ifremer. Available at: http://archimer.ifremer.fr/doc/00256/36691/ [Accessed December 19, 2016].

Fuentes J, Reyero I, Zapata C & Alvarez G (1994). Production traits of the mussel *Mytilus galloprovincialis* cultured in Galicia (NW of Spain): relative effects of source of seed and growing environment. *Aquaculture* **122,** 19–31.

Gaitán-Espitia JD, Quintero-Galvis JF, Mesas A & D'Elía G (2016). Mitogenomics of southern hemisphere blue mussels (Bivalvia: Pteriomorphia): Insights into the evolutionary characteristics of the *Mytilus edulis* complex. *Sci Rep* **6**, 26853. doi: 10.1038/srep26853.

Garnier M, Labreuche Y, Garcia C, Robert M & Nicolas J-L (2007). Evidence for the involvement of pathogenic bacteria in summer mortalities of the Pacific oyster *Crassostrea gigas*. *Microb Ecol* **53,** 187–196. doi: 10.1007/s00248-006-9061-9

Gay M, Berthe F & Le Roux F (2004). Screening of *Vibrio* isolates to develop an experimental infection model in the Pacific oyster *Crassostrea gigas*. *Dis Aquat Organ* **59,** 49–56. doi: 10.3354/dao059049

Glize P & Gourmelen G (2018). *Suivi de la mortalité de populations de moules en Pays de la Loire : Programme TESCOR 2*. SMIDAP & CRC des Pays de la Loire. Available at: https://www.smidap.fr/Table/Actualites/. [Accessed May 24, 2016].

Gosling E (1992). *The mussel Mytilus: ecology, physiology, genetics and culture*, Elsevier Science. E. Gosling, Amsterdam. ISBN: 0-444-88752-0

Gosling E (2003). *Bivalve Molluscs: Biology, Ecology and Culture*, Fishing News Books. E. Gosling, Oxford. ISBN: 978-0-85238-234-9

Goudenège D, Travers MA, Lemire A, Petton B, Haffner P, Labreuche Y, Tourbiez D, Mangenot S, Calteau A, Mazel D, Nicolas JL, Jacq A & Le roux F (2015). A single regulatory gene is sufficient to alter *Vibrio aestuarianus* pathogenicity in oysters: Comparative genomics of *Vibrio aestuarianus*. *Environ Microbiol* **17,** 4189–4199. doi: 10.1111/1462-2920.12699

Goulletquer P, Soletchnik P, Le Moine O, Razet D, Geairon P, Faury N & Taillade S (1998). Summer mortality of the Pacific cupped oyster *Crassostrea gigas* in the Bay of Marennes Oléron (France). In *ICES Mariculture Committee CM1998/CC:14 Theme Session: Population Biology*, pp. 14–21. Available at: http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.334.3103&rep=rep1&type=pdf [Accessed June 25, 2019].

Kumar S, Stecher G & Tamura K (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol Biol Evol* **33,** 1870–1874. doi: 10.1093/molbev/msw054

Lemire A, Goudenège D, Versigny T, Petton B, Calteau A, Labreuche Y & Le Roux F (2015). Populations, not clones, are the unit of *vibrio* pathogenesis in naturally infected oysters. *ISME J* **9,** 1523–1531. doi: 10.1038/ismej.2014.233

Lupo C et al. (2019). *Revue systématique de la littérature relative aux facteurs de risque de mortalité des moules exploitées en France*. Ifremer / ANSES. Available at: https://hal.archives-ouvertes.fr/hal-02087720/. [Accessed May 24, 2019].

Macián MC, Garay E, González-Candelas F, Pujalte MJ & Aznar R (2000). Ribotyping of *Vibrio* Populations Associated with Cultured Oysters (*Ostrea edulis*). *Syst Appl Microbiol* **23,** 409–417. doi: 10.1016/S0723-2020(00)80072-7

Mallet AL, Carver CEA, Coffen SS & Freeman KR (1987). Mortality Variations in Natural Populations of the Blue Mussel, *Mytilus edulis*. *Can J Fish Aquat Sci* **44,** 1589–1594. doi: 10.1139/f87-192

Mallet AL, Freeman KR & Dickie LM (1986). The genetics of production characters in the blue mussel *Mytilus edulis*. I. A preliminary analysis. *Aquaculture* **57,** 133–140.

Mallet AL & Haley LE (1984). General and specific combining abilities of larval and juvenile growth and viability estimated from natural oyster populations. *Mar Biol* **81,** 53–59. doi: 10.1007/BF00397625

Myrand B, Guderley H & Himmelman JH (2000). Reproduction and summer mortality of blue mussels *Mytilus edulis* in the Magdalen Islands, southern Gulf of St. Lawrence. *Mar Ecol Prog Ser* **197,** 193–207. doi: 10.3354/meps197193

Najdek M & Sapunar J (1987). Total and methyl-mercury content in bivalves, *Mytilus galloprovincialis* Lamarck and *Ostrea edulis* Linnaeus: Relationship of biochemical composition and body size. *Bull Environ Contam Toxicol* **39,** 56–62. doi: 10.1007/BF01691789

Nasfi H, Travers MA, de Lorgeril J, Habib C, Sannie T, Sorieul L, Gerard J, Avarre JC, Haffner P, Tourbiez D, Renault T, Furones D, Roque A, Pruzzo C, Cheslett D, Gdoura R & Vallaeys T (2015). A European epidemiological survey of *Vibrio splendidus* clade shows unexplored diversity and massive exchange of virulence factors. *World J Microbiol Biotechnol* **31,** 461–475. doi: 10.1007/s11274-015-1800-y

Oden E, Burioli EAV, Trancart S, Pitel PH & Houssin M (2016). Multilocus sequence analysis of *Vibrio splendidus* related-strains isolated from blue mussel *Mytilus* sp. during mortality events. *Aquaculture* **464,** 420–427. doi: 10.1016/j.aquaculture.2016.07.024

Ortigosa M, Esteve C & Pujalte M-J (1989). *Vibrio* Species in Seawater and Mussels: Abundance and Numerical Taxonomy. *Syst Appl Microbiol* **12,** 316–325. doi: 10.1016/S0723-2020(89)80080-3

Parisi M-G, Li H, Jouvet LBP, Dyrynda EA, Parrinello N, Cammarata M & Roch P (2008). Differential involvement of mussel hemocyte sub-populations in the clearance of bacteria. *Fish Shellfish Immunol* **25,** 834–840. doi: 10.1016/j.fsi.2008.09.005

Peperzak L & Poelman M (2008). Mass mussel mortality in The Netherlands after a bloom of Phaeocystis globosa (prymnesiophyceae). *J Sea Res* **60,** 220–222. doi: 10.1016/j.seares.2008.06.001

Pfeffer CS, Hite MF & Oliver JD (2003). Ecology of *Vibrio vulnificus* in Estuarine Waters of Eastern North Carolina. *Appl Environ Microbiol* **69,** 3526–3531. doi: 10.1128/AEM.69.6.3526-3531.2003

Philipp EER, Kraemer L, Melzner F, Poustka AJ, Thieme S, Findeisen U, Schreiber S & Rosenstiel P (2012). Massively Parallel RNA Sequencing Identifies a Complex Immune Gene Repertoire in the lophotrochozoan *Mytilus edulis*. *PLOS ONE* **7,** e33091. doi: 10.1371/journal.pone.0033091

Polsenaere P, Soletchnik P, Le Moine O, Gohin F, Robert S, Pépin J-F, Stanisière J-Y, Dumas F, Béchemin C & Goulletquer P (2017). Potential environmental drivers of a regional blue mussel mass mortality event (winter of 2014, Breton Sound, France). *J Sea Res* **123,** 39–50. doi: 10.1016/j.seares.2017.03.005

Prou JM & Goulletquer P (2002). The French Mussel Industry: Present Status and Perspectives. *Bull Aquac Assoc Can* **103,** 17–23.

Riquet F, Simon A & Bierne N (2017). Weird genotypes? Don't discard them, transmissible cancer could be an explanation. *Evol Appl* **10,** 140–145. doi: 10.1111/eva.12439

Romero A, Costa M, Forn-Cuni G, Balseiro P, Chamorro R, Dios S, Figueras A & Novoa B (2014). Occurrence, seasonality and infectivity of *Vibrio strains* in natural populations of mussels *Mytilus galloprovincialis*. *Dis Aquat Organ* **108,** 149–163. doi: 10.3354/dao02701

Seed R & Suchanek TH (1992). Population and community ecology of *Mytilus*. In *The mussel Mytilus: ecology, physiology, genetics and culture*, Elsevier Science., Developments in aquaculture and fisheries science, pp. 87–169. E. Gosling, Amsterdam. ISBN: 0-444-88752-0

Snieszko SF (1974). The effects of environmental stress on outbreaks of infectious diseases of fishes*. *J Fish Biol* **6,** 197–208. doi: 10.1111/j.1095-8649.1974.tb04537.x

Soletchnik P, Le Moine O, Faury N, Razet D, Geairon P & Goulletquer P (1999). Mortalité de l'huître *Crassostrea gigas* dans le bassin de Marennes-Oléron : étude de la variabilité spatiale de son environnement et de sa biologie par un système d'informations géographiques (SIG). *Aquat Living Resour* **12,** 131–143. Available at: https://archimer.ifremer.fr/doc/1999/publication-862.pdf [Accessed July 8, 2019].

Soletchnik P & Robert S (2016). *Eléments de connaissance sur la mortalité et la reproduction de la moule bleue (Mytilus edulis) sur la façade atlantique*. Ifremer. Available at: https://archimer.ifremer.fr/doc/00345/45634/45263.pdf [Accessed February 12, 2017].

Tamura K, Stecher G, Peterson D, Filipski A & Kumar S (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol Biol Evol* **30,** 2725–2729. doi: 10.1093/molbev/mst197

Tanguy M, McKenna P, Gauthier-Clerc S, Pellerin J, Danger J-M & Siah A (2013). Functional and molecular responses in *Mytilus edulis* hemocytes exposed to bacteria, *Vibrio splendidus*. *Dev Comp Immunol* **39,** 419–429. doi: 10.1016/j.dci.2012.10.015

Travers M-A, Boettcher Miller K, Roque A & Friedman CS (2015). Bacterial diseases in marine bivalves. *J Invertebr Pathol* **131,** 11–31. doi: 10.1016/j.jip.2015.07.010

Travers M-A, Pepin J-F, Soletchnik P, Guesdon S & Le Moine O (2016). *Mortalités de moules bleues dans les Pertuis Charentais – MORBLEU*. Ifremer. Available at: http://archimer.ifremer.fr/doc/00324/43539/ [Accessed December 19, 2016].

Travers M-A, Tourbiez D, Parizadeh L, Haffner P, Kozic-Djellouli A, Aboubaker M, Koken M, Dégremont L & Lupo C (2017). Several strains, one disease: experimental investigation of *Vibrio aestuarianus* infection parameters in the Pacific oyster, *Crassostrea gigas*. *Vet Res* **48**:32. doi: 10.1186/s13567-017-0438-1.

Tsuchiya M (1983). Mass mortality in a population of the mussel *Mytilus edulis* L. caused by high temperature on rocky shores. *J Exp Mar Biol Ecol* **66,** 101–111. doi: 10.1016/0022-0981(83)90032-1

Venier P, Varotto L, Rosani U, Millino C, Celegato B, Bernante F, Lanfranchi G, Novoa B, Roch P, Figueras A & Pallavicini A (2011). Insights into the innate immunity of the

656 Mediterranean mussel *Mytilus galloprovincialis*. *BMC Genomics* **12,** 69. doi:
657 10.1186/1471-2164-12-69

658

7. Table(s)

Table 1: Bacterial load of pure bacterial suspension (BL) used to make the dilutions used for each bacterial challenge, and number of infected mussels, total dead mussels and strain-positive dead mussels in each condition tested for every monthly experiment. S1: *Vibrio crassostreae* 7T4_12 and S2: *Vibrio splendidus* 3G1_6; C1: dilution 1:10 (10^{-1}) and C2: dilution 1:10,000 (10^{-4}).

	BL (CFU mL^{-1})		***number of infected mussels / dead mussels / strain-positive dead mussels***				
Months	**S1**	**S2**	**S1_C1**	**S1_C2**	**S2_C1**	**S2_C2**	**Negative controls**
Apr 2017	7.22 x 10^8	NA[§]	100 / 18 / 15	100 / 0 / 0	NA[§]	NA	100 / 0 / 0
May 2017	2.50 x 10^9	NA	100 / 9 / 4	100 / 0 / 0	NA	NA	100 / 0 / 0
Jun 2017	5.64 x 10^8	NA	100 / 0 / 0	100 / 0 / 0	NA	NA	100 / 4 / 0
Jul 2017	5.85 x 10^9	NA	100 / 1 / 0	100 / 0 / 0	NA	NA	100 / 1 / 0
Aug 2017	1.19 x 10^9	9.27 x 10^9	100 / 0 / 0	100 / 0 / 0	100 / 16 / 9	100 / 0 / 0	100 / 0 / 0
Sep 2017	3.09 x 10^9	1.41 x 10^9	100 / 0 / 0	100 / 0 / 0	100 / 12 / 11	100 / 0 / 0	100 / 0 / 0
Oct 2017	1.13 x 10^9	3.00 x 10^8	100 / 9 / 7	100 / 0 / 0	100 / 31 / 23	100 / 0 / 0	100 / 1 / 0
Nov 2017	6.27 x 10^9	4.27 x 10^9	100 / 2 / 2	100 / 0 / 0	100 / 4 / 4	100 / 0 / 0	100 / 0 / 0
Dec 2017	5.18 x 10^8	2.82 x 10^9	100 / 5 / 0	100 / 2 / 0	100 / 0 / 0	100 / 1 / 0	100 / 4 / 0
Jan 2018	7.91 x 10^9	4.45 x 10^8	100 / 23 / 4	100 / 8 / 0	100 / 9 / 1	100 / 11 / 1	100 / 5 / 0
Feb 2018	8.73 x 10^9	4.09 x 10^8	100 / 39 / 18	100 / 3 / 0	100 / 86 / 34	100 / 3 / 0	100 / 4 / 0
Mar 2018	1.49 x 10^9	4.73 x 10^8	100 / 65 / 30	100 / 10 / 0	100 / 86/ 34	100 / 1 / 0	100 / 0 / 0
Apr 2018	6.36 x 10^8	6.36 x 10^8	100 / 18 / 10	100 / 3 / 1	100 / 82 / 47	100 / 3 / 0	100 / 1 / 0
	m[†] = 3.12 x 10^9 ($\pm$ 3.00 x 10^9)	**m[†] = 2.23 x 10^9** ($\pm$ 2.93 x 10^9)	**Σ[‡] = 1,300 / 189 / 90**	**Σ = 1,300 / 26 / 1**	**Σ = 900 / 326 / 163**	**Σ = 900 / 19 / 1**	**Σ = 1,300 / 20 / 0**

[§] no data; [†] mean; [‡] sum

8. Figures

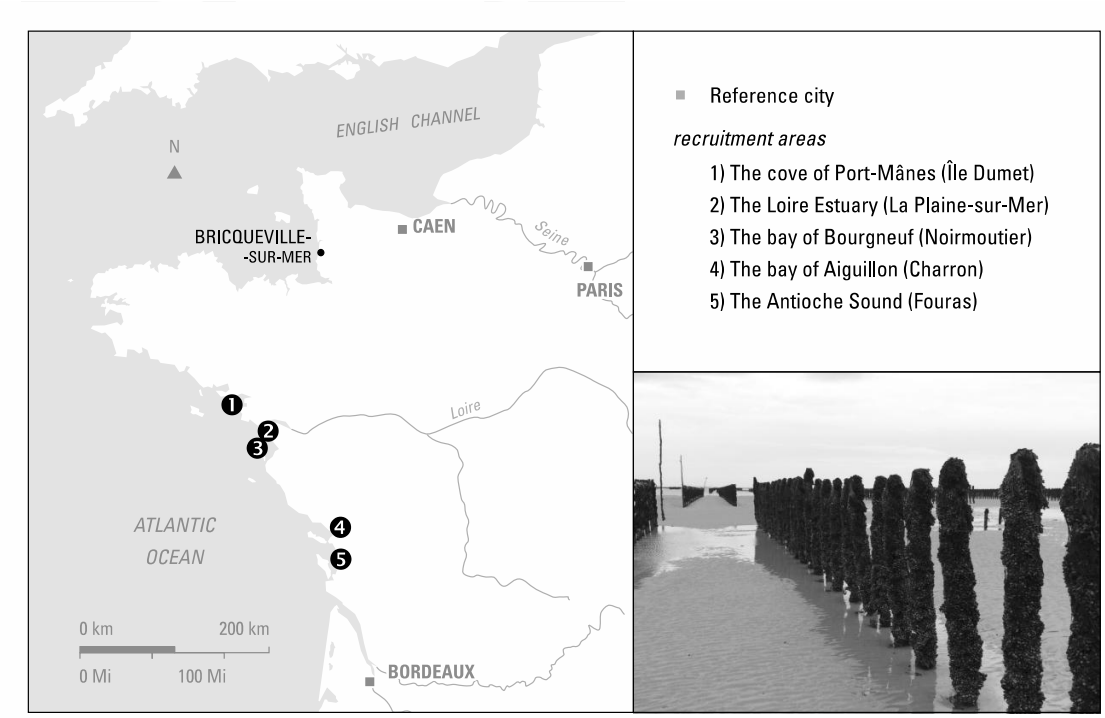


Figure 1: Location of the five mussel original recruitment areas and the shellfish growing area Bricqueville-sur-Mer (Normandy, France) with a picture of '*bouchot*'.

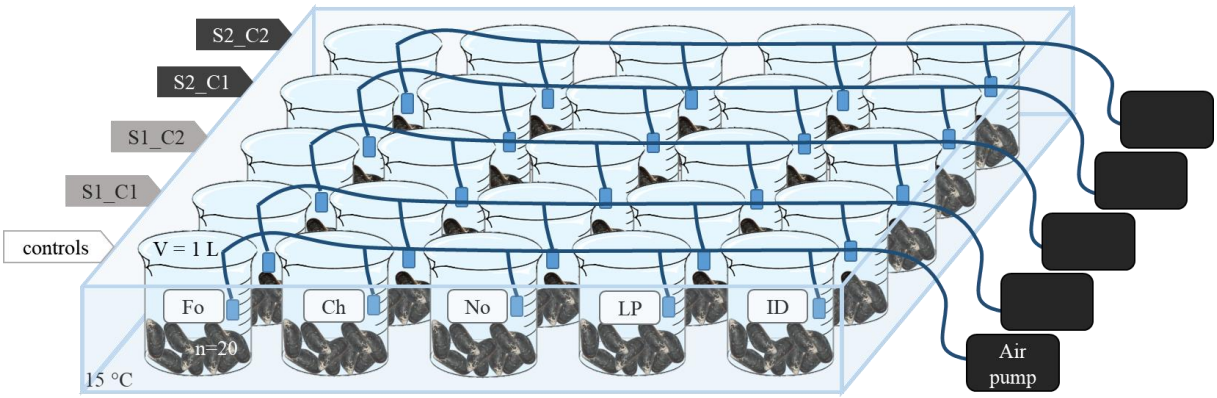
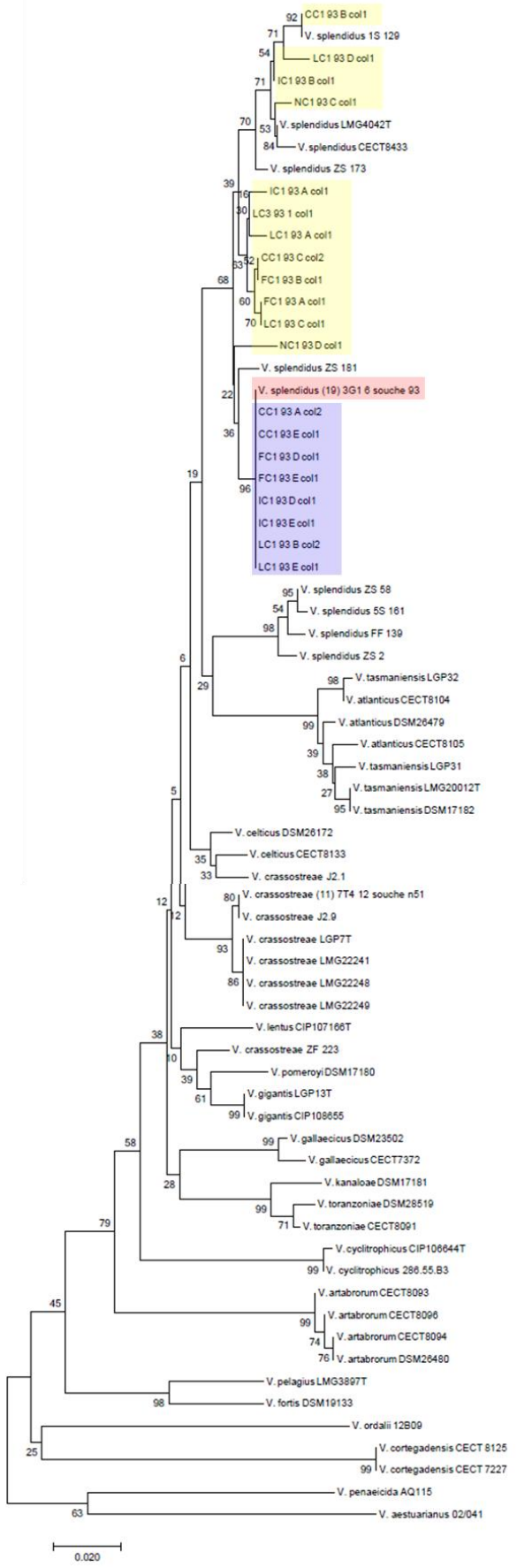
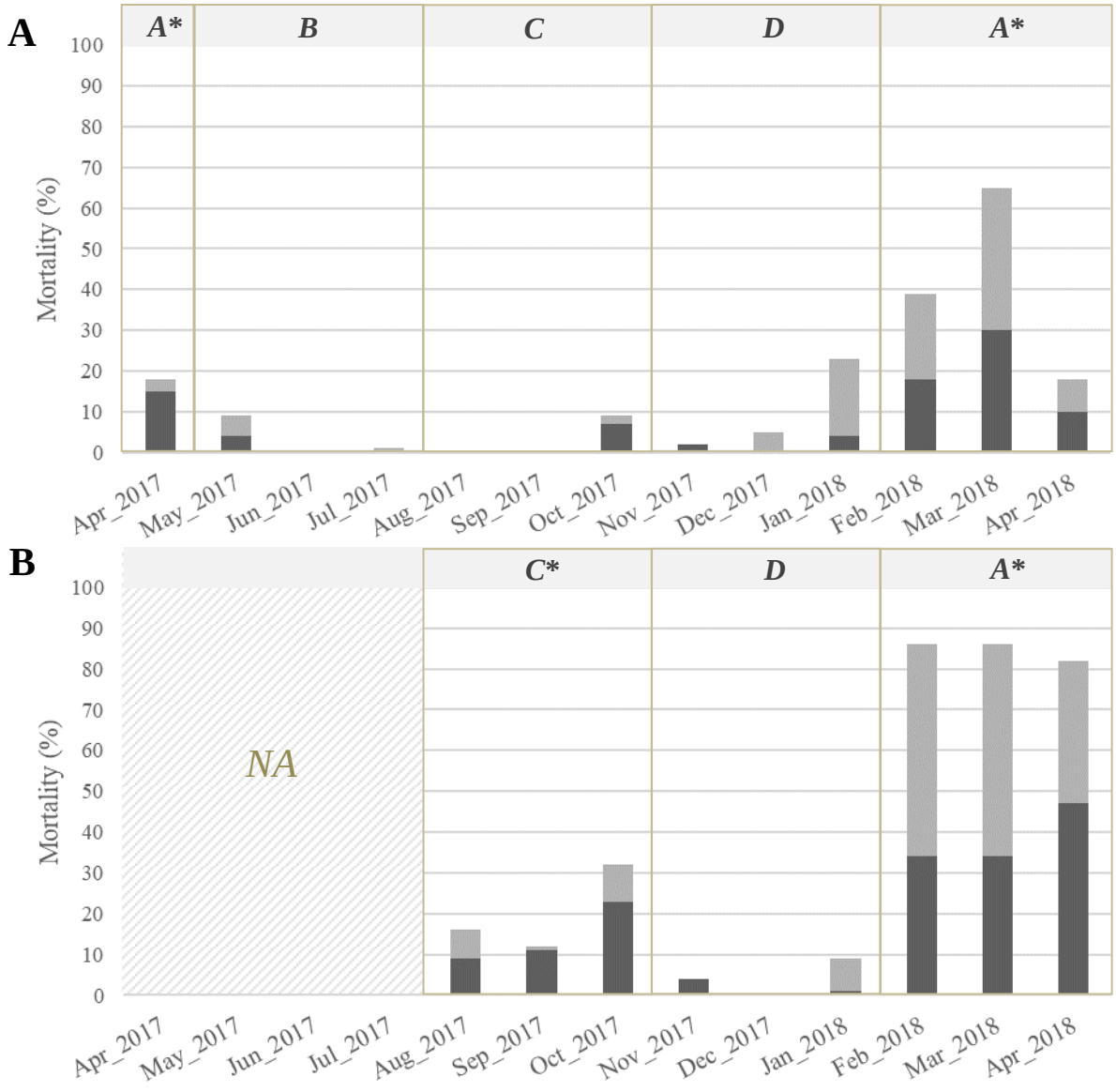


Figure 2: Schematisation of the post-infection monitoring system. Mussels inoculated with 100 µL of artificial sterilised sea water (controls), dilution 1:10 (C1) or dilution 1:10,000 (C2) of pure bacterial suspension of *Vibrio crassostreae* 7T4_12 (S1) or *Vibrio splendidus* 3G1_6 (S2).

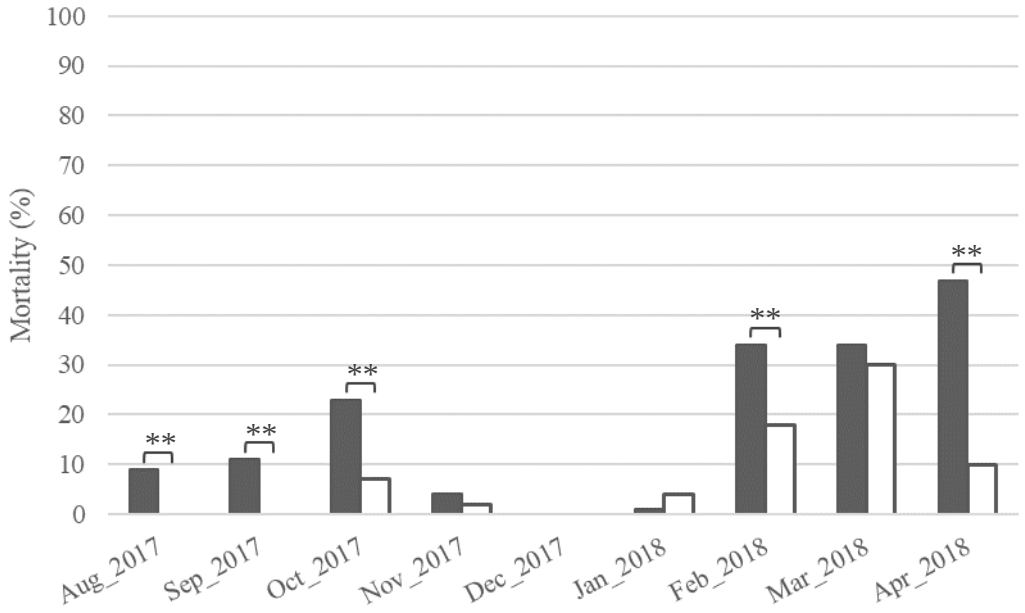

CC1 93 B col1
V. splendidus 1S 129
LC1 93 D col1
IC1 93 B col1
NC1 93 C col1
V. splendidus LMG4042T
V. splendidus CECT8433
V. splendidus ZS 173
IC1 93 A col1
LC3 93 1 col1
LC1 93 A col1
CC1 93 C col2
FC1 93 B col1
FC1 93 A col1
LC1 93 C col1
NC1 93 D col1
V. splendidus ZS 181
V. splendidus (19) 3G1 6 souche 93
CC1 93 A col2
CC1 93 E col1
FC1 93 D col1
FC1 93 E col1
IC1 93 D col1
IC1 93 E col1
LC1 93 B col2
LC1 93 E col1
V. splendidus ZS 58
V. splendidus 5S 161
V. splendidus FF 139
V. splendidus ZS 2
V. tasmaniensis LGP32
V. atlanticus CECT8104
V. atlanticus DSM26479
V. atlanticus CECT8105
V. tasmaniensis LGP31
V. tasmaniensis LMG20012T
V. tasmaniensis DSM17182
V. celticus DSM26172
V. celticus CECT8133
V. crassostreae J2.1
V. crassostreae (11) 7T4 12 souche n51
V. crassostreae J2.9
V. crassostreae LGP7T
V. crassostreae LMG22241
V. crassostreae LMG22248
V. crassostreae LMG22249
V. lentus CIP107166T
V. crassostreae ZF 223
V. pomeroyi DSM17180
V. gigantis LGP13T
V. gigantis CIP108655
V. gallaecicus DSM23502
V. gallaecicus CECT7372
V. kanaloae DSM17181
V. toranzoniae DSM28519
V. toranzoniae CECT8091
V. cyclitrophicus CIP106644T
V. cyclitrophicus 286.55.B3
V. artabrorum CECT8093
V. artabrorum CECT8096
V. artabrorum CECT8094
V. artabrorum DSM26480
V. pelagius LMG3897T
V. fortis DSM19133
V. ordalii 12B09
V. cortegadensis CECT 8125
V. cortegadensis CECT 7227
V. penaeicida AQ115
V. aestuarianus 02/041
0.020

672

Figure 3: Phylogenic tree made from *mreB* sequences of *Vibrio spendidus*-related strains isolated during one of the experiments (color box) and forty-eight different reference bacterial strains (Oden *et al.*, 2016); *Vibrio aestuarianus* 02/041 and *Vibrio penaeicida* AQ115, were provided by the Genomic of *Vibrio* Research Department (CNRS Roscoff, France) and used as *Splendidus* clade outsider. Horizontal branch lengths are proportional to evolutionary divergence. Bootstrap scores of 1000 replicates appear next to the corresponding branch (significant bootstrap score > 80). In color boxes, *mreB* sequences of (red box) *Vibrio splendidus* 3G1_6 (S2), (blue box) S2-positive dead mussels and (yellow box) S2-negative dead mussels.

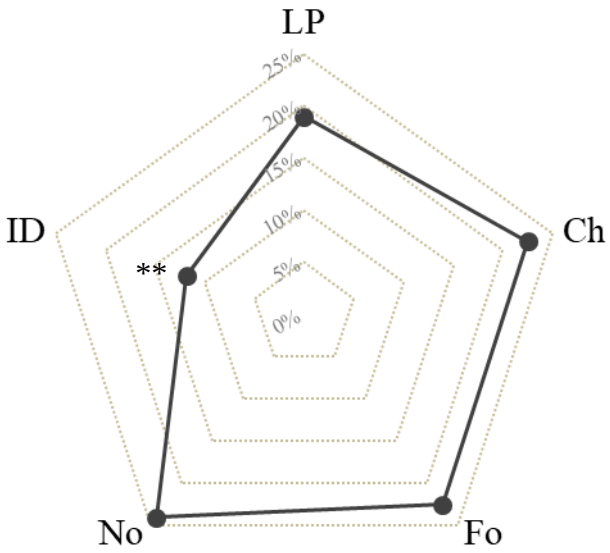


683 **Figure 4:** Mortality (%) observed in each monthly experiment at the highest concentration (10^7
684 CFU/mussel). A: experimental infection with *Vibrio crassostreae* 7T4_12 (S1); B: experimental
685 infection with *Vibrio splendidus* 3G1_6 (S2). Dark grey bars: S-positive dead mussels in injected
686 strain; light grey bars: negative dead mussels in injected strain; grey bars (light & dark): 'all' dead
687 mussels. At the top of the histograms are indicated the periods of the year (A, B, C and D) defined in
688 *2.5*. Kruskal-Wallis test and Wilcoxon test; p-value* < 0.05. NA: no data.
689



690

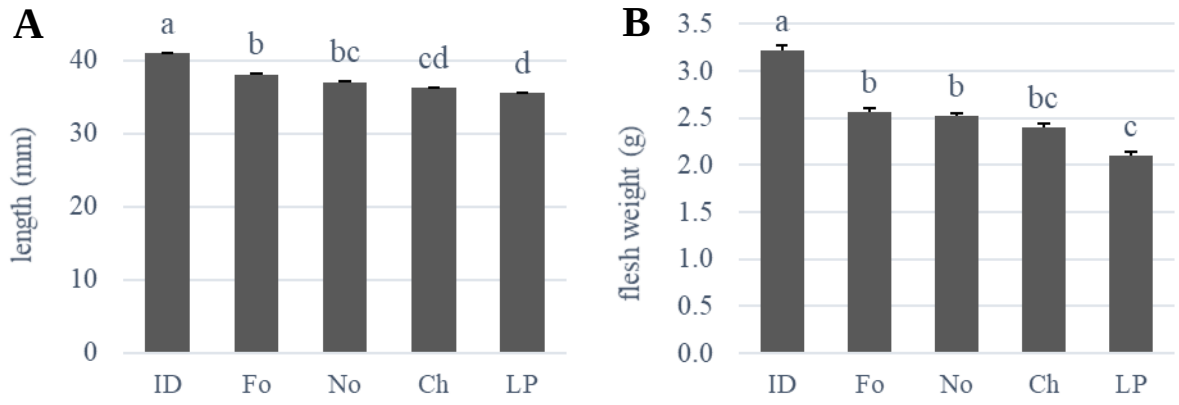
691 **Figure 5:** Comparison of mortalities obtained at each month with *Vibrio crassostreae* 7T4_12 (white
692 bars) and *Vibrio splendidus* 3G1_6 (dark grey bars) at the highest concentration (10^8 CFU mL^{-1}). Chi-
693 squared test of independence; p-value** < 0.01.



694

695 **Figure 6:** Proportion (%) of dead mussels on all experiments (all strains included) according to the
696 origin of mussels. ID: Ile Dumet; LP: La-Plaine-sur-Mer; Ch: Charron; Fo: Fouras; No: Noirmoutier.
697 Chi-squared; p-value** < 0.01.

698



699

700 **Figure 7:** Comparison of the length (mm) (A) and flesh weight (g) (B) of dead mussels according to
701 their origin. ID: Ile Dumet; LP: La-Plaine-sur-Mer; Ch: Charron; Fo: Fouras; No: Noirmoutier. Values
702 are means ± SEM. ANOVA, p-value** < 0.01 and Tukey's *post hoc* test. The similarity among the
703 bars is emphasised through a letter system, placed above them; bars that do not share the same letter
704 are significantly different.

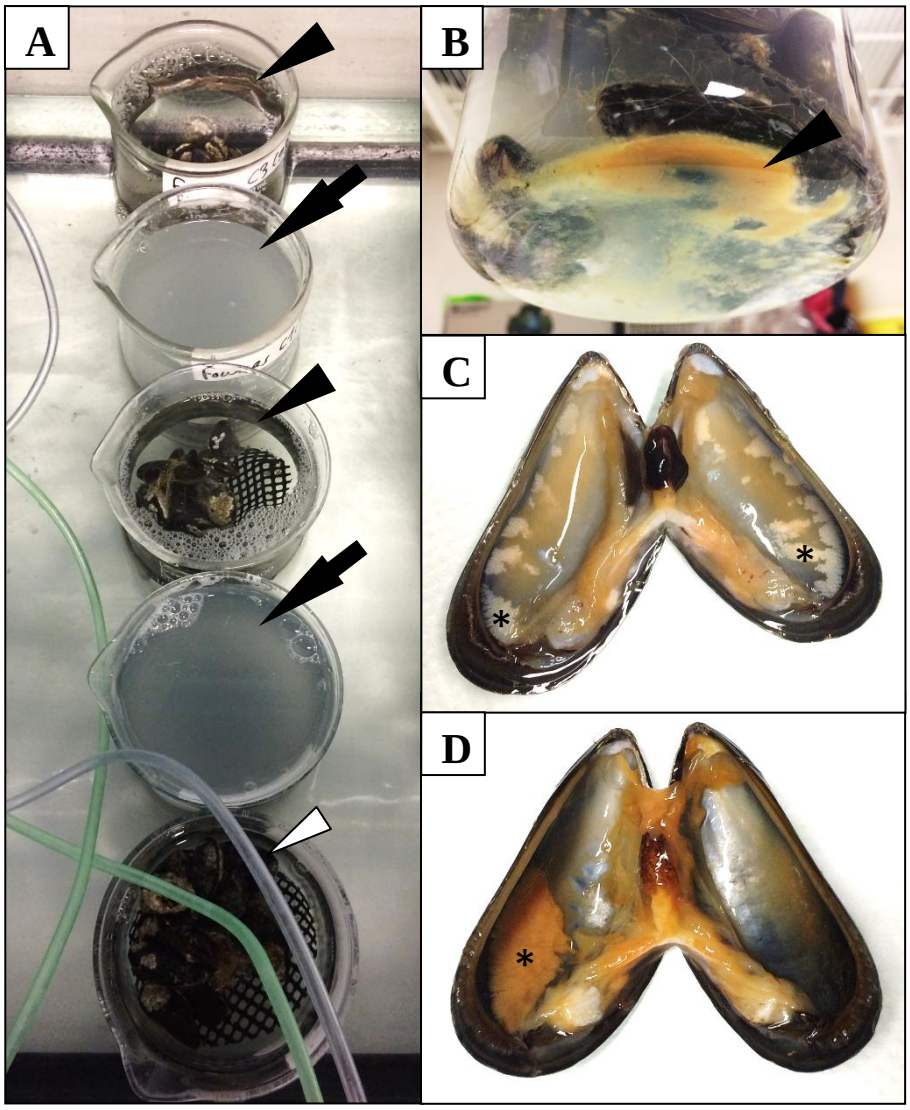


Figure 8: Spawning observed during experiments conducted through period A (Feb. to Apr.). (A) Male egg-laying in tanks where mussels were infected with both strains *Vibrio crassostreae* 7T4_12 and *Vibrio splendidus* 3G1_6 at the highest concentration (10^8 CFU mL^{-1}) (black arrows). No spawning in negative controls (white arrowhead), or in mussels infected with both strains at the lowest concentration (10^5 CFU mL^{-1}) (black arrowheads). (B) Female egg-laying sediment in tanks where mussels were infected with both strains at the highest concentration. Dead male (C) and female (D) mussel with remnants of gametes not expelled into the mantle (un-spawned gametes) (*).