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

4

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6

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## 10 **Abstract**

11           This study aimed at assessing the pathogenicity of two *Vibrio splendidus*-related  
12 species and evaluating the influence of the origin and annual life cycle of mussels on their  
13 sensitivity during a bacterial challenge. Thus, *in vivo* infection assays were made with *Vibrio*  
14 *crassostreae* 7T4\_12 and *Vibrio splendidus* 3G1\_6, over respectively thirteen and nine  
15 months, on adult blue mussels from five recruitment areas in France. Two bacterial  
16 concentrations were tested: one consistent with the loads of *Vibrio* spp. in environment and  
17 mussel tissues ( $\sim 10^5$  CFU mL<sup>-1</sup>) and another one much higher ( $\sim 10^8$  CFU mL<sup>-1</sup>). The tested  
18 environmental concentration has no pathogenic effect whatever the time of year, the strain  
19 used and the origin of mussels. However, at the highest concentration, a pathogenic effect was  
20 observed only at specific moments, and one of the origins appeared to be more resistant. The  
21 physiological state of mussels –depending on the time of year– seemed significant in mussels’  
22 sensitivity, as their recruitment origin. This study is the first to test the pathogenicity of *V.*  
23 *splendidus*-related strains at concentrations close to what is found in the wild, over the annual  
24 cycle of mussels, and considering their origin.

25

### 26 **Keywords:**

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

## 28 1. Introduction

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

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

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

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

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

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

78 different recruitment areas, by using two *V. splendidus*-related species described as virulent  
79 for mussels from Brittany at high concentrations (Le Roux, pers. comm., unpubl. data): *Vibrio*  
80 *crassostreae* 7T4\_12 and *V. splendidus* 3G1\_6. The aims of this study were to evaluate (i) the  
81 pathogenicity and the virulence of these two strains on mussels by testing two different  
82 concentrations: one consistent with the general environmental loads of *Vibrio* spp. in seawater  
83  $\sim 10^4/10^5$  CFU mL<sup>-1</sup> (Pfeffer *et al.*, 2003; Eiler *et al.*, 2007), also corresponding to the  
84 bacterial concentrations that can be reached in *M. edulis* (equal to  $2.9 \times 10^4$  CFU per mL or g  
85 according to Garnier *et al.*, 2007), and another one much higher  $\sim 10^8$  CFU mL<sup>-1</sup>; (ii) the  
86 influence of the first farming method used and original spat recruitment area on the sensitivity  
87 to vibriosis; (iii) the role of physiological and morphological conditions of mussels –impacted  
88 by the season– in susceptibility to bacterial infections.

## 89 **2. Materials and Methods**

### 90 **2.1 Bacterial strains and inoculum preparation**

91 The two *V. splendidus*-related strains used for experimental infection were provided from the  
92 French institute, Ifremer. As part of their study (ANR 13-ADAP-0007-01 “OPOPOP”), they  
93 isolated many strains from a *Vibrio* population assembly isolated from oyster tissues (*C.*  
94 *gigas*) during and outside mortality events that hit oysters and mussels in the bay of Brest  
95 (Finistère, France) in 2014. Among the hundred species isolated, several appeared to be  
96 highly pathogenic for oysters (Bruto *et al.*, 2017) and mussels from Brittany (Le Roux, pers.  
97 comm., unpubl. data) of which *V. crassostreae* 7T4\_12 and *V. splendidus* 3G1\_6, that are  
98 used in this study, and respectively named S1 and S2 in the rest of the article. Twenty-four  
99 hours before infection, the bacteria were grown in fresh liquid sterile marine Zobell at 22 °C.  
100 The bacterial inocula were prepared using the fresh bacterial suspensions by diluting it in  
101 Artificial Sterilised Sea Water (ASSW) from 1:10 to 1:10,000,000 (serial ten-fold dilutions  
102 from  $10^{-1}$  to  $10^{-7}$ ). The two dilutions used as inocula for experimental infections were 1:10

103 ( $10^{-1}$ ) and 1:10,000 ( $10^{-4}$ ). Bacterial load of pure bacterial suspension (BL) was defined by  
104 enumeration of Colony Forming Units (CFU) according to the standard NF ISO7218:2007  
105 (AFNOR, 2007):  $BL (CFU mL^{-1}) = \frac{\Sigma c}{V \times 1,1 \times d}$ ; where  $\Sigma c$  is the sum of the colonies counted on  
106 the two plates of marine Zobell agar spread with two successive dilutions ( $10^{-6}$  and  $10^{-7}$ ) that  
107 incubated for 48 h at 22 °C;  $V$  is the volume that has been spread on plate (0.1 mL) and  $d$  is  
108 the dilution corresponding to the first dilution retained ( $10^{-6}$ ).

## 109 **2.2 Experimental mussels**

110 Adult mussels used for this experiment were placed on intertidal mussel stakes (*'bouchot'*) in  
111 the shellfish growing area off the coast of Bricqueville-sur-Mer, Normandy, France  
112 ( $48^{\circ}55'05.8''N$   $1^{\circ}34'23.9''W$ ) since autumn 2015. Spats were initially collected in early  
113 spring 2015 in five different recruitment areas: the cove of Port-Mânes (Ile Dumet, Loire-  
114 Atlantique, France); the Loire Estuary (La-Plaine-sur-Mer, Loire-Atlantique, France); the bay  
115 of Bourgneuf (Noirmoutier, Vendée, France); the bay of Aiguillon (Charron, Charente-  
116 Maritime, France); the Antioche Sound (Fouras, Charente-Maritime, France) (Fig. 1). These  
117 five origin groups are respectively named ID, LP, No, Ch, Fo in the rest of the article. The  
118 mussels from ID were different from the others: first, larval recruitment and first months of  
119 farming were made on longlines (while the others were on *'bouchot'*). Second, they were  
120 *'pelisse'* mussels, *i.e.* mussels from the upper layer of the collection rope that grew faster than  
121 the others. To leave space for the lower layer mussels, and to prevent these bundles of  
122 mussels from coming off, farmer collects and puts them back into 2.5 m long mesh tubes.  
123 Like initial collection rope, these nets are then coiled around a wooden pole at the growing  
124 area.

## 125 **2.3 Experimental procedure of bacterial challenges**

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

128 collected during low tide, transported in cooler bags to the laboratory and once there,  
129 immediately put back in tanks filled with oxygenated natural seawater at 15 °C overnight. The  
130 next day, they were anaesthetised by bathing in a solution containing 40 g L<sup>-1</sup> of magnesium  
131 chloride during 30 min. Then, 100 µL of inocula or ASSW (for negative controls) per mussel  
132 were injected into the posterior adductor muscle. In the rest of the article, the bacterial loads  
133 corresponding to the 100 µL of dilutions 10<sup>-1</sup> and 10<sup>-4</sup> have been respectively named C1 and  
134 C2. Following the injections, 20 mussels of each group (origin/strain/loads) were placed in  
135 separated tanks filled with 1 L of aerated seawater at 15 °C (Fig. 2). Thus, 100 mussels were  
136 injected for each condition (S1\_C1; S1\_C2; S2\_C1; S2\_C2 or negative controls). Water  
137 exchange and tank cleaning were carried daily for seven days and then, mortalities were  
138 recorded. All mussels that did not spontaneously close their valves when the seawater was  
139 removed were considered as dead. Dead mussels were sampled for biometry (flesh weight and  
140 shell length) and microbiological analysis. The objective was to verify whether among the  
141 main bacteria found in the tissues of dead mussels, the strain initially injected was present  
142 (Koch's fourth postulate).

#### 143 **2.4 Microbiological and molecular analyses of dead mussels**

144 Tissues of dead mussels were mashed with scalpels and a volume of 450 µL of ASSW was  
145 added to 50 mg of the homogenate. After stirring and a pulse centrifugation to pellet cellular  
146 debris, ten-fold dilutions of supernatant were made and 100 µL of 1: 10,000 (10<sup>-4</sup>) and 1:  
147 100,000 (10<sup>-5</sup>) dilutions were sown on Zobell marine agar and incubated for 48 h at 22 °C.  
148 For each plate, the three predominant bacterial colonies were re-isolated in order to ensure  
149 their purity. Then, DNA extraction of these three predominant colonies was performed by  
150 heating the colony placed in 250 µL of purified water (DNA/nuclease free water) for 10 min  
151 at 95 °C. The successful extraction was confirmed by DNA quantification with NanoDrop<sup>TM</sup>  
152 2000c spectrophotometer (ThermoFisher Scientific<sup>TM</sup>, Waltham, MA USA) and strains were



153 then characterised by molecular analysis. One TaqMan<sup>®</sup> real-time PCR targeting the *16S*  
154 *rRNA* gene of *V. splendidus*-related strains (PCR1) (Oden *et al.*, 2016) was carried out on a  
155 Smart Cycler<sup>®</sup> (Cepheid, USA); the primers used for PCR1 were *SpF1*  
156 5'ATCATGGCTCAGATTGAACG3' and *SpR1* 5'CAATGGTTATCCCCACATC3' (Nasfi  
157 *et al.*, 2015) and the probe *SpProbe* (5'-3') FAM-CCCATTAACGCACCCGAAGGATTG-  
158 BHQ1. The reaction volume of 25 µL contained 12.5 µL of Premix Ex Taq<sup>®</sup> 2 X Takara<sup>®</sup>  
159 (Lonza, Verviers, Belgium), 0.5 µL of each primer (20 µM), 0.5 µL of probe (10 µM), 9 µL  
160 of purified water and 2 µL of extracted DNA (replaced with 2 µL of purified water in the  
161 negative control). The thermal cycling profile was 95 °C for 10 s, followed by 40 cycles at 95  
162 °C for 5 s and 62 °C for 30 s. Then, when PCR1 was positive, the objective was to  
163 differentiate strains of *V. splendidus*-related species from each other and identify the  
164 inoculated strain.

## 165 **2.5 Genotyping of *V. splendidus*-related strains**

166 A conventional PCR targeting the housekeeping genes *mreB* of *V. splendidus*-related strains  
167 (PCR2) was performed since it is the most discriminant gene for the identification of closely  
168 related strains among the *Splendidus* clade (Oden *et al.*, 2016); the primer pair for PCR2 was  
169 *mreB*-F 5'CTGGTGCTCGYGAGGTTTAC3' and *mreB*-R  
170 5'CCRTTYTCTGAKATATCAGAAGC3'. For the conventional PCR, typical 25 µL reaction  
171 mixtures contained 12.5 µL of Premix Ex Taq<sup>®</sup> 2 X Takara<sup>®</sup> (Lonza, Verviers, Belgium), 0.5  
172 µL of each primer (20 µM), 9.5 µL of purified water and 2 µL of DNA template (replaced  
173 with 2 µL of purified water in the negative control). Conventional PCR amplifications were  
174 carried out in a T100<sup>™</sup> Thermal Cycler (Bio-Rad, France) and the thermal program was as  
175 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  
176 extension of 3 min at 72 °C. PCR products were then analysed with QIAxcel<sup>®</sup> Advanced

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

## 179 **2.6 Phylogenetic analyses**

180 The *mreB* sequences were aligned using a multiple sequence alignment Multiple Sequence  
181 Comparison by Log-Expectation (MUSCLE); phylogenetic analyses were performed in  
182 MEGA7 (Kumar *et al.*, 2016) using the Neighbor Joining method (Tamura *et al.*, 2013) and  
183 the maximum composite likelihood model with a bootstrap of 1000 replications. Thus, the  
184 sequences of the *mreB* gene of *V. splendidus*-related strains from dead mussels could be  
185 compared with the sequences of the injected strains and with forty-seven different reference  
186 bacterial strains (whose forty-two belonging to the *Splendidus* clade; see 2.1. in Oden *et al.*  
187 2016). *Vibrio aestuarianus* 02/041, *Vibrio ordalii* 12B09 and *Vibrio penaeicida* AQ115, were  
188 provided from Genomic of *Vibrio* Research Department (CNRS Roscoff, France) and used as  
189 *Splendidus* clade outsiders.

## 190 **2.7 Establishment of periods related to the annual life cycle of mussels**

191 In order to estimate the role of physiological conditions of mussels in susceptibility to  
192 bacterial infections, ‘periods’ have been defined, related to the mussel cycle. The cycle of *M.*  
193 *edulis* in cold waters has been known for several years (Boromthanarat *et al.* , 1987; Gosling,  
194 2003): gametogenesis begins in late summer and early autumn (Sept./Oct.), gonads are ripe  
195 and animals mature in early winter (Dec./Jan.). The emission of gametes in water occurs in  
196 late winter and early spring (Feb.-Apr.) and the resting stage begins in late spring and early  
197 summer (June). Thus, the following periods have been defined: from February to April =  
198 period A; from May to July = period B; from August to October = period C; from November  
199 to January = period D.

## 200 **2.8 Statistical analyses**

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

## 215 **3. Results**

### 216 **3.1 Bacterial inocula concentrations**

217 The BL of all experiments performed with S1 and S2 are reported in Table 1. Mussels were  
218 inoculated with average bacterial loads equal to  $3.12 \times 10^7$  CFU (S1\_C1) and  $3.12 \times 10^4$  CFU  
219 (S1\_C2) for S1, and  $2.23 \times 10^7$  CFU (S2\_C1) and  $2.23 \times 10^4$  CFU (S2\_C2) for S2.

### 220 **3.2 Overall obtained mortalities and strain-positive mussels**

221 The numbers of infected mussels, dead mussels and strain-positive dead mussels obtained for  
222 each experimental infection are reported in Table 1. On all experimental infections made with  
223 S1, 1,300 mussels were inoculated with S1\_C1 and 189 died (mean = 15; min = 0; max = 65),  
224 and 1,300 mussels were infected with S1\_C2 and 26 died (mean = 2; min = 0; max = 10). On

225 all bacterial challenges performed with S2, 900 mussels were inoculated with S2\_C1 and 326  
226 died (mean = 36; min = 0; max = 86), and 900 mussels were infected with S2\_C2 and 19 died  
227 (mean = 2; min = 0; max = 11). On average over all experimentations, S1 was found in 48 %  
228 (90/189) of dead mussels in condition S1\_C1 and in 4 % (1/26) in S1\_C2, and S2 was found  
229 in 50 % (163/326) of dead mussels in condition S2\_C1 and in 5 % (1/19) in S2\_C2. None of  
230 the dead mussels in the negative controls (n = 15) were positive in S1 or S2. Figure 3 shows  
231 one of the phylogenetic analysis; in total, 255 *mreB* sequences were aligned over all these  
232 bacterial challenges.

### 233 3.2.1 Comparison of mortality by tested conditions (strains / loads)

234 The observed mortality between the tested conditions differed significantly (K-W, S1: p-  
235 value\*\* =  $2.7 \cdot 10^{-5}$ ; S2: p-value\*\* =  $2.1 \cdot 10^{-6}$ ). For S1 and S2, the mortality obtained at the  
236 highest concentration (C1) is significantly higher from the others (Wilcoxon test, S1: C1/C2  
237 p-value\* = 0.02 and C1/controls p-value\*\* = 0.009; S2: C1/C2 p-value\*\* = 0.007 and  
238 C1/controls p-value\*\* = 0.0006). There was no difference between C2 and negative controls  
239 for both strains (positive to the strain or not).

### 240 3.2.2 Comparison of mortality by period

241 Figure 4 illustrates the obtained mortality with S1 (Fig. 4A) and S2 (Fig. 4B) at each month of  
242 each period at the highest concentration tested (C1). The obtained mortality differs according  
243 to the defined periods and therefore according to the life cycle of mussels (K-W, S1: p-value\*  
244 = 0.04; S2: p-value\* = 0.02). For S1, period A is significantly different from periods B, C and  
245 D (Wilcoxon test, p-values\* < 0.05). For S2, there is no difference between period A and C  
246 (Wilcoxon test, p-value = 0.07) but significant difference exists between, A or C, and period  
247 D (Wilcoxon test, p-values\* < 0.05). What is also quite remarkable in this figure is the  
248 percentage of S-pos dead mussels; over the period A, the percentage of S-pos dead mussels  
249 was on average < 50 % for S1 and S2 while for period C, it was ~ 75 % for S2.

### 250 3.2.3 Comparison of the pathogenicity of the two strains over the common time interval

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

### 260 3.2.4 Comparison of mortality depending on spat recruitment sites

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

## 265 3.3 Biometry

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

## 271 3.4 Other observations

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

275 observed in more than a third of dead mussels. Also, less than 10 % of the mussels had their  
276 mantle full of macroscopically visible trematode sporocysts or metacercaria.  
277 For experiments done during period A, an interesting phenomenon was observed. Just a few  
278 hours after injection, the mussels that have been infected at the highest concentration with S1  
279 and S2, began to lay their gametes in water tank (Fig. 8A and Fig. 8B). When spawning was  
280 observed, the water was immediately changed. However, this occurred several times during  
281 the first 2-3 days after infection. For dead mussels collected during this period, more than half  
282 had a mantle with some remnants of gametes not expelled (un-spawned gametes) (Fig. 8C and  
283 Fig. 8D) or totally empty (like the valve on the right in Fig. 8D).

## 284 **4. Discussion**

### 285 **4.1 Pathogenicity of *V. crassostreae* 7T4\_12 and *V. splendidus* 3G1\_6**

286 Performing an enumeration to obtain the BL of bacterial suspension of each inoculum  
287 allowed to verify precisely the tested concentrations. By achieving the objective of testing  
288 bacterial concentrations consistent with those found in seawater or in mussel tissues ( $\sim 10^4$   
289 CFU animal<sup>-1</sup>; Eiler *et al.*, 2007; Garnier *et al.*, 2007; Pfeffer *et al.*, 2003), as well as high  
290 concentrations ( $\sim 10^7$  CFU animal<sup>-1</sup>), different results was observed. Most of the experimental  
291 infections performed on mussels (Parisi *et al.*, 2008; Tanguy *et al.*, 2013; Ben Cheikh *et al.*,  
292 2016, 2017) or oysters (Gay *et al.*, 2004; Garnier *et al.*, 2007; Travers *et al.*, 2017) were made  
293 by injections with high bacterial strain concentrations around  $10^7/10^8$  CFU animal<sup>-1</sup> which is  
294 quite far from those found in the field. On this point, Travers *et al.* (2017) mentioned that  
295 defining a strain as virulent through high concentration injections was a debatable issue. As  
296 for Goudenège *et al.* (2015), they determined three categories of strains as part of their high-  
297 throughput bacterial infections: virulent ( $> 50$  % mortalities at  $10^2$  CFU animal<sup>-1</sup>), non-  
298 virulent ( $< 50$  % mortalities at  $10^7$  CFU animal<sup>-1</sup>) and intermediate (*i.e.* pathogenic; an effect  
299 is observed only at  $10^7$  CFU animal<sup>-1</sup>). They have thereby shown that a real virulent strain

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

## 325 **4.2 Influence of the annual life cycle during a bacterial challenge**

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



350 Sapunar, 1987; Myrand *et al.*, 2000). The results observed here are consistence with these  
351 studies. Indeed, the high mortalities obtained during period A were the outcome of global  
352 weakness of mussels –that accompanies this triggering of spawning– and the inoculation of a  
353 high concentration of pathogenic bacteria. It is even more obvious because, as for S1, no  
354 mortality has been observed during other periods. Inoculation induced significant stress that  
355 triggered spawning; it is then possible to wonder: if the nature of the stress had been different  
356 (*e.g.* chemical contaminant spill or toxic algae bloom), would the spawning have been  
357 triggered and what would have been the result on the observed mortality rates? Nevertheless,  
358 in the case of S2, the strain seems to have increased mortality more strongly during this period  
359 than for S1, but as showed above, S2 caused mortality during period C while S1 did not. This  
360 is interesting because this post-spawning period corresponds to the sexual rest during which  
361 glycogen content is improved and is accumulated as an energetic reserves (Bayne & Worrall,  
362 1980); it shows that mussels remain sensitive during this period and this is also what Myrand  
363 *et al.* (2000) observed in their analysis of the link between reproduction and summer mortality  
364 in Québec mussels. On the other hand, period D appears to be the interval when mussels are  
365 more robust since no significant mortality has occurred. During this period, energy reserves  
366 are usually used to meet the energy needs of gametogenesis and in December, mussels are full  
367 –mature– with gonads that occupy all the space, without being ready or able to spawn. The  
368 lack of observed mortality suggests that they have accumulated enough energy to fight against  
369 and deal with high infection. It seems that this time of the year is the most appropriate time to  
370 test a strain for virulence or pathogenicity; indeed, if strain causes mortality at that moment,  
371 then it will cause probably more at another time.

### 372 **4.3 Mortality according to original spat recruitment site**

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

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

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

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

400 viability is highly heritable in *M. edulis* populations and, very recently, Dégremont *et al.*  
401 (2019) revealed that selected populations of French *M. edulis* were significantly more  
402 resistant to mortality outbreaks than natural unselected populations. *Mytilus edulis* and *M.*  
403 *galloprovincialis* are native species to the French coast, and numerous different natural  
404 populations exist and are capable to hybridise with each other. Thus, the Atlantic coasts of  
405 France is very complex –in terms of genetic structure– with several mosaic hybrid zone  
406 between *M. edulis* and *M. galloprovincialis* (Bierne *et al.*, 2003; Riquet *et al.*, 2017). It is  
407 therefore possible that some natural French populations could be more resistant than others.

408

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

417

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

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

429

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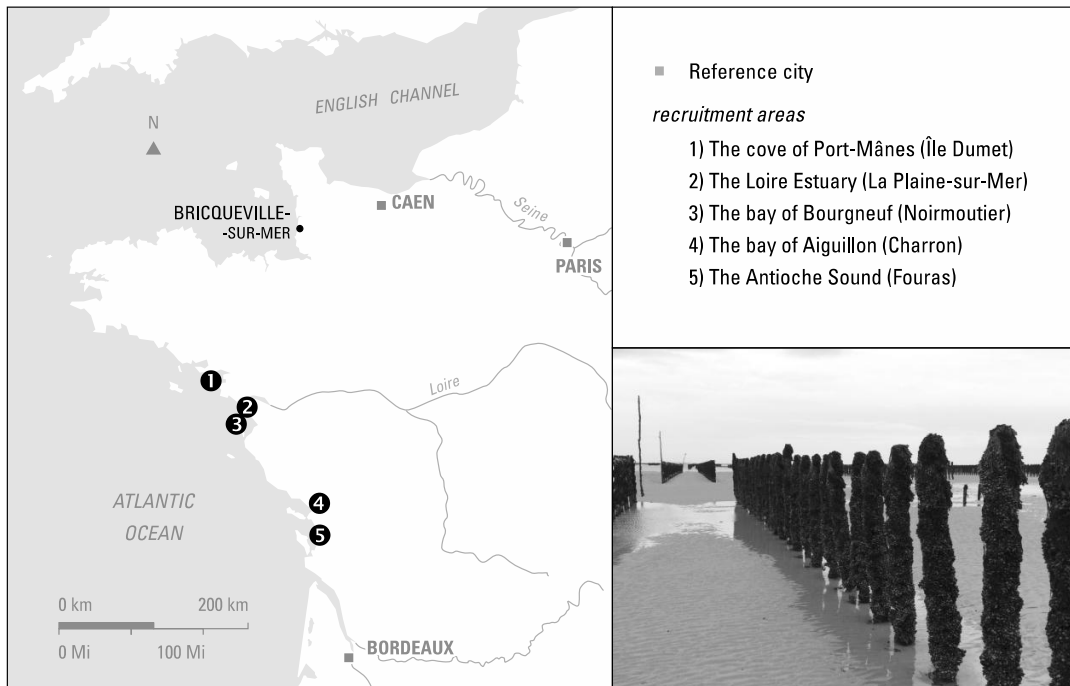
659 **7. Table(s)**

660 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  
661 dead mussels and strain-positive dead mussels in each condition tested for every monthly experiment. S1: *Vibrio crassostreae* 7T4\_12 and S2: *Vibrio*  
662 *splendidus* 3G1\_6; C1: dilution 1:10 ( $10^{-1}$ ) and C2: dilution 1:10,000 ( $10^{-4}$ ).

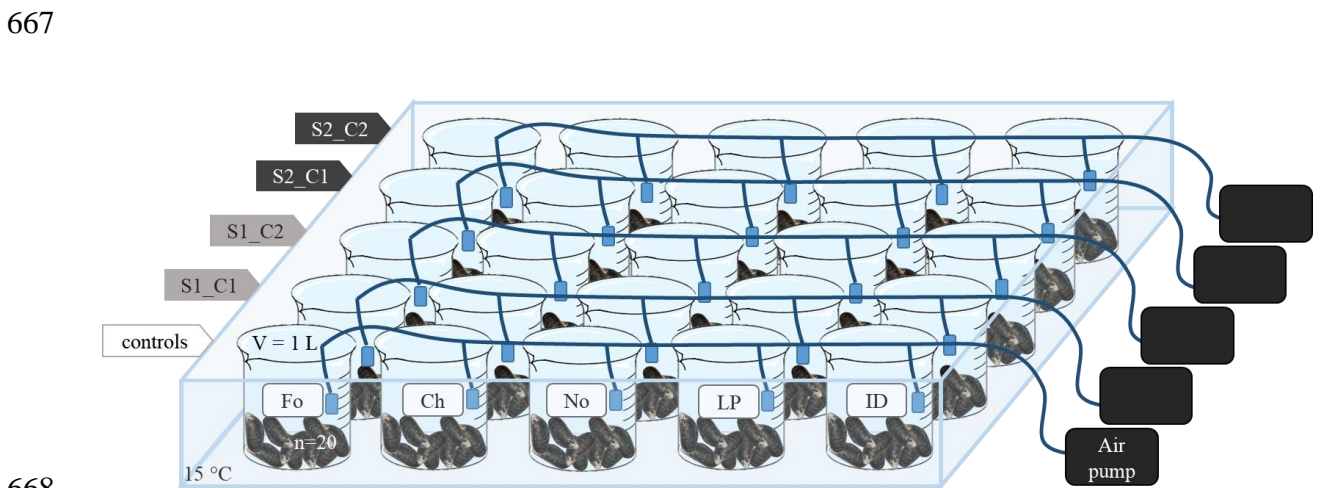
Months	<i>BL (CFU mL<sup>-1</sup>)</i>		<i>number of infected mussels / dead mussels / strain-positive dead mussels</i>				
	S1	S2	S1_C1	S1_C2	S2_C1	S2_C2	Negative controls
Apr 2017	7.22 x 10 <sup>8</sup>	NA <sup>§</sup>	100 / 18 / 15	100 / 0 / 0	NA <sup>§</sup>	NA	100 / 0 / 0
May 2017	2.50 x 10 <sup>9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

§ no data; † mean; ‡ sum

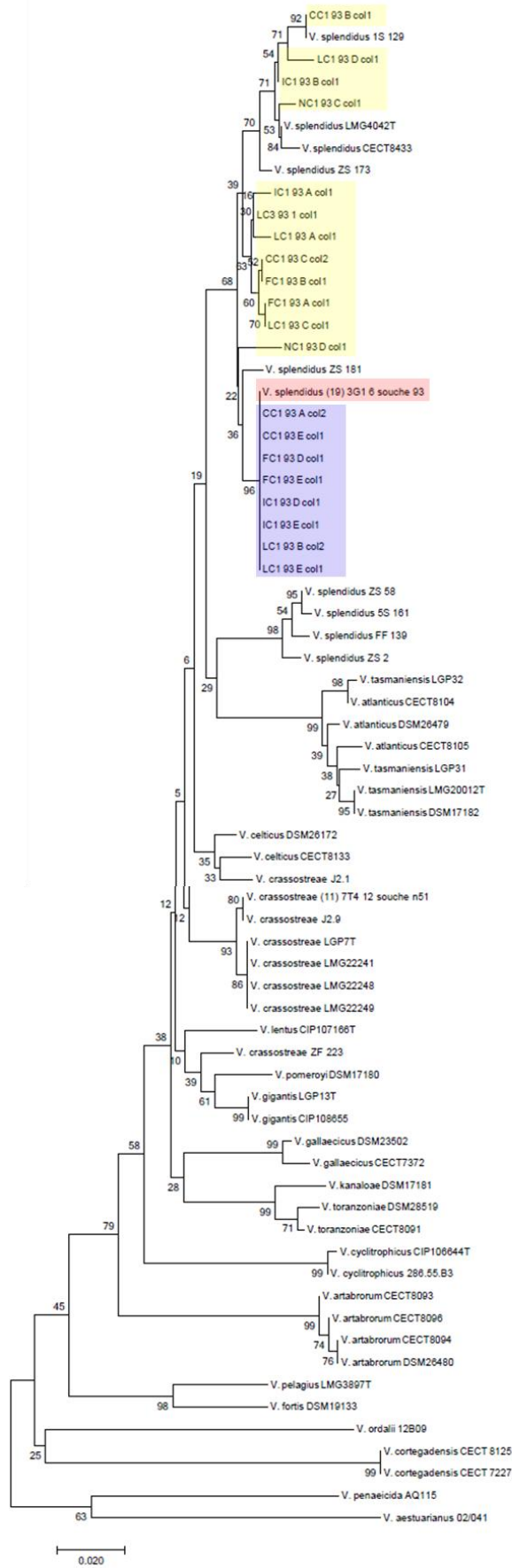
663 **8. Figures**



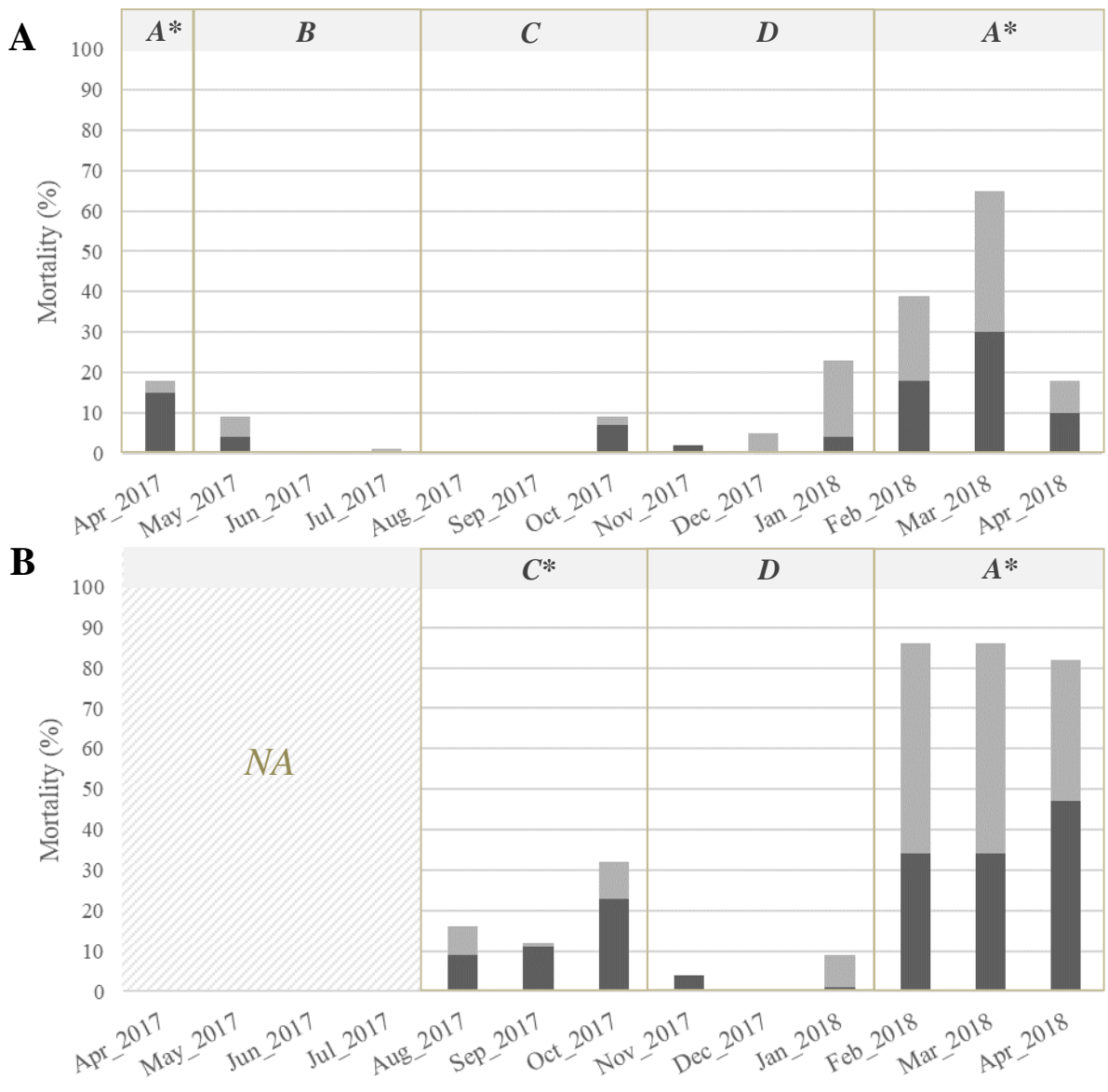
664  
 665 **Figure 1:** Location of the five mussel original recruitment areas and the shellfish growing area  
 666 Bricqueville-sur-Mer (Normandy, France) with a picture of ‘bouchot’.



668  
 669 **Figure 2:** Schematisation of the post-infection monitoring system. Mussels inoculated with 100  $\mu$ L of  
 670 artificial sterilised sea water (controls), dilution 1:10 (C1) or dilution 1:10,000 (C2) of pure bacterial  
 671 suspension of *Vibrio crassostreae* 7T4\_12 (S1) or *Vibrio splendidus* 3G1\_6 (S2).



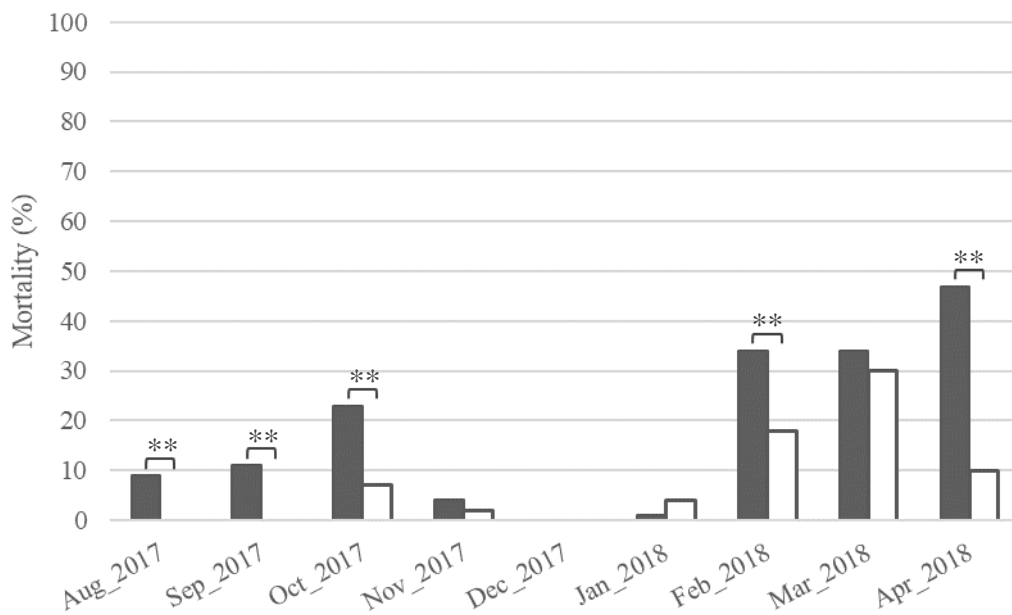
673 **Figure 3:** Phylogenetic tree made from *mreB* sequences of *Vibrio splendidus*-related strains isolated  
 674 during one of the experiments (color box) and forty-eight different reference bacterial strains (Oden *et*  
 675 *al.*, 2016); *Vibrio aestuarianus* 02/041 and *Vibrio penaeicida* AQ115, were provided by the Genomic  
 676 of *Vibrio* Research Department (CNRS Roscoff, France) and used as *Splendidus* clade outsider.  
 677 Horizontal branch lengths are proportional to evolutionary divergence. Bootstrap scores of 1000  
 678 replicates appear next to the corresponding branch (significant bootstrap score > 80). In color boxes,  
 679 *mreB* sequences of (red box) *Vibrio splendidus* 3G1\_6 (S2), (blue box) S2-positive dead mussels and  
 680 (yellow box) S2-negative dead mussels.  
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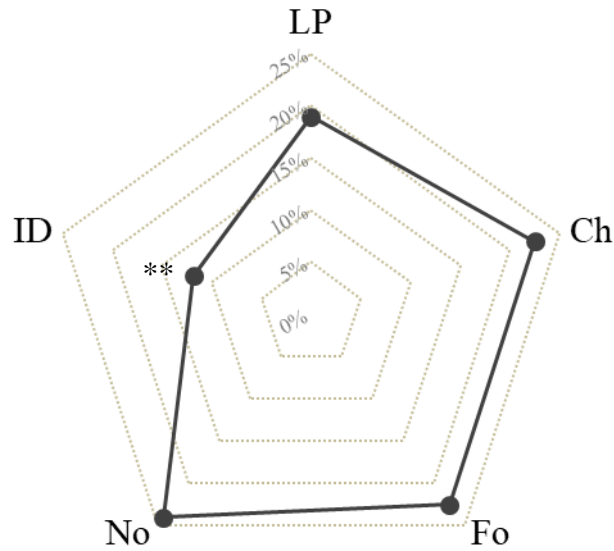
683 **Figure 4:** Mortality (%) observed in each monthly experiment at the highest concentration ( $10^7$   
 684 CFU/mussel). A: experimental infection with *Vibrio crassostreae* 7T4\_12 (S1); B: experimental  
 685 infection with *Vibrio splendidus* 3G1\_6 (S2). Dark grey bars: S-positive dead mussels 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.

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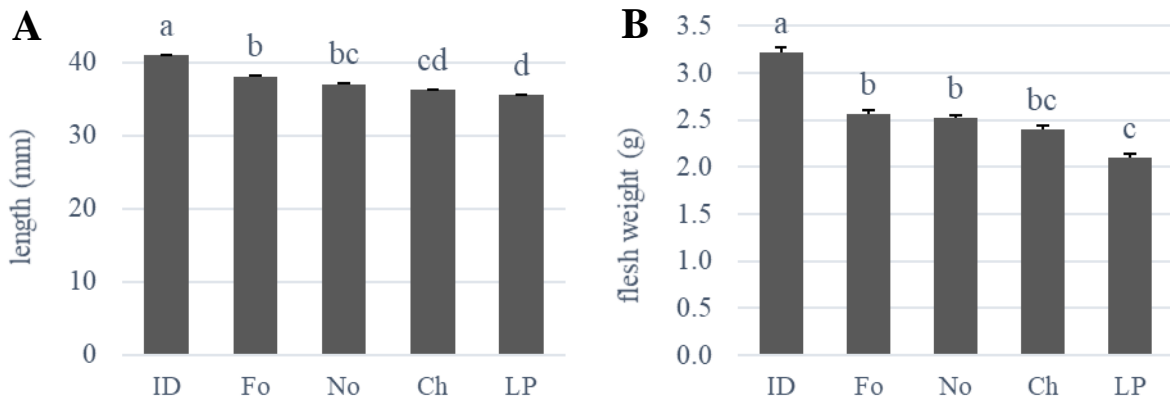
691 **Figure 5:** Comparison of mortalities obtained at each month with *Vibrio crassostreae* 7T4\_12 (white  
 692 bars) and *Vibrio splendidus* 3G1\_6 (dark grey bars) at the highest concentration ( $10^8$  CFU mL<sup>-1</sup>). Chi-  
 693 squared test of independence; p-value\*\* < 0.01.



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.

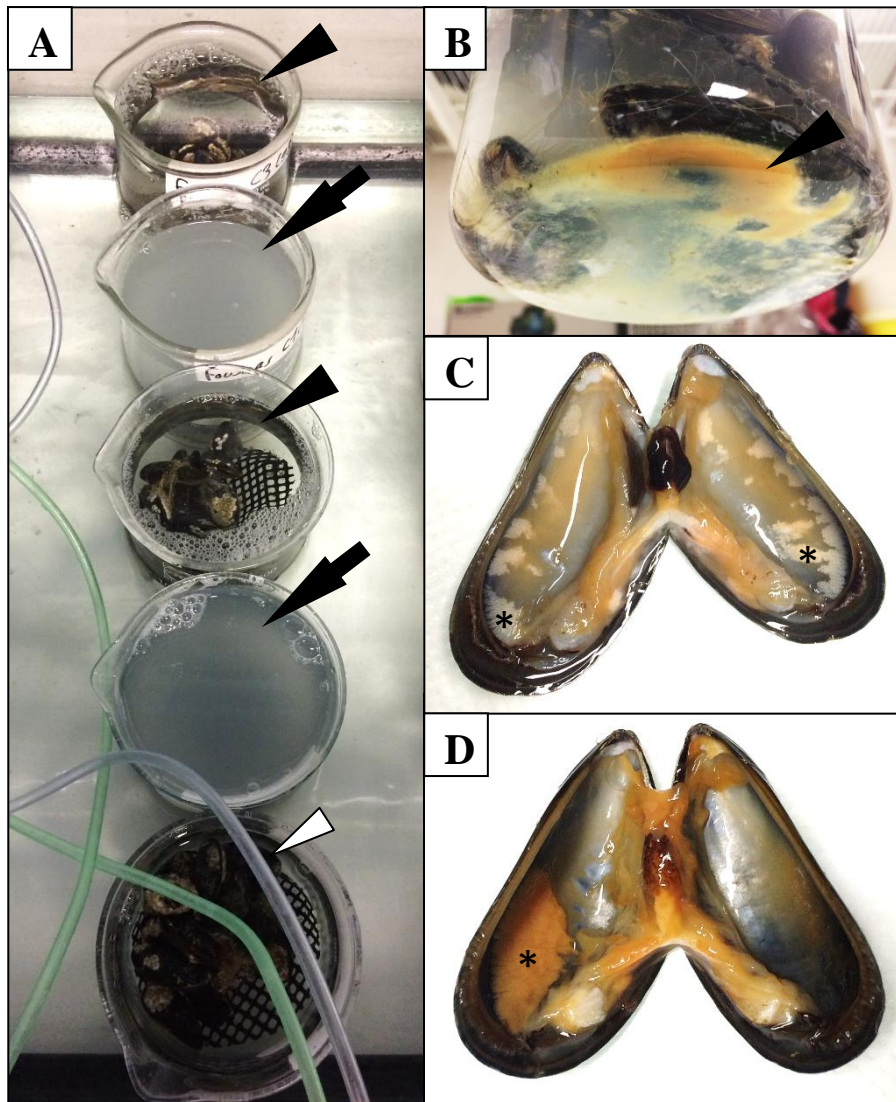
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699

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  $\pm$  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.





705

706 **Figure 8:** Spawning observed during experiments conducted through period A (Feb. to Apr.). (A)  
 707 Male egg-laying in tanks where mussels were infected with both strains *Vibrio crassostreae* 7T4\_12  
 708 and *Vibrio splendidus* 3G1\_6 at the highest concentration ( $10^8$  CFU mL<sup>-1</sup>) (black arrows). No  
 709 spawning in negative controls (white arrowhead), or in mussels infected with both strains at the lowest  
 710 concentration ( $10^5$  CFU mL<sup>-1</sup>) (black arrowheads). (B) Female egg-laying sediment in tanks where  
 711 mussels were infected with both strains at the highest concentration. Dead male (C) and female (D)  
 712 mussel with remnants of gametes not expelled into the mantle (un-spawned gametes) (\*).

713