



Aquatic Ecology of the Oyster Pathogens *Vibrio splendidus* and  
*Vibrio aestuarianus*

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1 Aquatic Ecology of the Oyster Pathogens *Vibrio splendidus* and  
2 *Vibrio aestuarianus*

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22 **Running title:** Ecology of *Vibrio splendidus* and *Vibrio aestuarianus*

49 **Abstract**

50 The ecology of the oyster pathogens *Vibrio splendidus* and *Vibrio aestuarianus* in the  
51 brackish aquatic environment was extensively investigated in this study. By conducting  
52 laboratory experiments under natural setting conditions it was shown that *V. splendidus*  
53 LGP32 strain generally exhibits longer persistence in both seawater and sediment than  
54 *V. aestuarianus* 01/32 strain. Both strains maintained viability and culturability for  
55 longer times in the sediment suggesting this compartment may represent a suitable  
56 niche for their persistence in the environment. In addition, both strains attached to chitin  
57 particles and copepods, the efficiency of attachment being higher in *V. splendidus* than  
58 in *V. aestuarianus*. Similarly, LGP32 strain showed a greater capability to form biofilm  
59 on PVC surfaces than 01/32 strain. LGP32 and 01/32 strains were also capable of  
60 entering a viable but nonculturable state after extended incubation at 5°C, a condition  
61 commonly found during cold season in the aquatic brackish environment. These results  
62 are consistent with field data collected during a two-year sampling campaign in the  
63 Northern Adriatic Sea, and provide background information on the mechanisms  
64 promoting *V. splendidus* and *V. aestuarianus* persistence in coastal water thus  
65 contributing to a better understanding of the epidemiology of the associated diseases.

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## 82 Introduction

83 Mollusk farming has become one of the largest aquaculture activity in the world. By  
84 volume, oysters (*Ostreidae*) are the second most important aquacultured taxonomic  
85 group to cyprinids at 4.5 million tons per year (FAO, 2013). In particular, the Pacific  
86 cupped oyster, *Crassostrea gigas*, provides the greatest contribution with 0.6 million  
87 tons per year of global production volume, valued at USD 1.3 billion in 2009 (FAO,  
88 2013).

89 Although mollusk culture is steadily growing in importance in the aquaculture sector,  
90 the European populations of cultivated mollusks are suffering from severe mortality  
91 outbreaks. From 2008, mass mortality episodes of *C. gigas* were reported in farming  
92 areas in several countries including France, Spain, the Netherlands, United Kingdom,  
93 Ireland, and the United States (Samain and McCombie, 2008). These outbreaks  
94 generally occur in summer in sheltered habitats when mortality can reach 80-100%  
95 especially in oyster juveniles. Causes triggering the outbreaks are still poorly  
96 understood and are attributed to complex interactions between oysters, pathogens and  
97 environmental parameters (Pernet et al., 2012).

98 The naturally occurring Gram-negative bacteria *Vibrio splendidus* and *Vibrio*  
99 *aestuarianus* have been associated to the summer mortalities affecting the production of  
100 *C. gigas* worldwide (Lacoste et al., 2001; Waechter et al., 2002; Le Roux et al., 2002;  
101 Gay et al., 2004a; Garnier et al., 2008). In particular, a *V. splendidus* LGP32 strain  
102 isolated from oysters suffering the “summer mortality syndrome” was shown to be  
103 highly pathogenic and cause death when injected to bivalves (Gay et al., 2004a,b; Le  
104 Roux et al., 2007). It was shown that *V. splendidus* LGP32 invades oyster immune cells,  
105 the hemocytes, through subversion of host-cell actin cytoskeleton and survives  
106 intracellularly by preventing acidic vacuole formation and limiting reactive oxygen  
107 species production (Duperthuy et al., 2010, 2011). In addition, a secreted  
108 metalloprotease (*vsm*) produced by this strain is associated with toxicity (Le Roux et al.,  
109 2007; Binesse et al., 2008). Epidemiological studies conducted during recurrent summer  
110 mortality events of *C. gigas* along the French Atlantic coast have also documented the  
111 predominance of another *Vibrio* strain, *V. aestuarianus*, that was isolated from the  
112 haemolymph of diseased animals and was related to mortality outbreaks in juvenile and  
113 adult oysters (Garnier et al., 2008; Saulnier et al., 2010). A zinc metalloprotease (*vam*)  
114 similar to that observed in *V. splendidus* is produced by this species and causes lethality

115 in *C. gigas* by impairing host cellular immune defenses (Labreuche *et al.*, 2010).  
116 Although *V. splendidus* and *V. aestuarianus* have been reported to be associated with  
117 summer mortality of Pacific cupped oyster spat and their interactions with bivalves have  
118 been investigated by a number of studies (Duperthuy *et al.*, 2010; Labreuche *et al.*,  
119 2010; Saulnier *et al.*, 2010, Duperthuy *et al.*, 2011), little is known on the ecology of  
120 these pathogens outside their bivalve host.

121 The lack of information on the aquatic ecology and lifestyle of these bacteria in coastal  
122 areas greatly limits our understanding of their role in the occurrence of mortality  
123 outbreaks and the epidemiology of such events. Vibrios are natural inhabitants of  
124 marine coastal and brackish environment throughout the world; they are equipped with  
125 a battery of adaptive response mechanisms which allow them to persist in the  
126 environment even during unfavorable conditions (*e.g.* temperature fluctuation, nutrient  
127 limitation, UV light stress, etc.) (Vezzulli *et al.*, 2009). Sea Surface Temperature (SST)  
128 is by far one of the main variables affecting the occurrence and viability of these  
129 bacteria in the aquatic environment as most vibrios show a strong seasonal oscillation  
130 with higher abundances generally observed when SST typically exceeds 18 °C (Vezzulli  
131 *et al.*, 2009). Below this temperature and especially at cold temperatures lower than 10  
132 °C, vibrios enter the viable but nonculturable (VBNC) state, a dormant condition in  
133 which cells remain viable, but are not culturable in conventional laboratory media (Xu  
134 *et al.*, 1982).

135 Several studies have clearly shown that attachment to surfaces is an integral part of the  
136 aquatic lifestyle of many vibrios, representing a successful survival mechanism  
137 (Vezzulli *et al.*, 2010). Animals with an exoskeleton of chitin, aquatic plants, protozoa,  
138 bivalves, waterbirds, as well as abiotic substrates (*e.g.* sediments) exemplify  
139 environmental matrices identified as preferential reservoirs for *Vibrio* bacteria (Pruzzo  
140 *et al.*, 2008; Vezzulli *et al.*, 2010). At molecular level, different bacterial colonization  
141 factors (*e.g.* pili and outer membrane and secreted proteins) have been described to  
142 promote *Vibrio* attachment to these substrates (Chiavelli *et al.*, 2001; Vezzulli *et al.*,  
143 2008; Stauder *et al.*, 2010). Adhesion represents the first step for substrate colonization  
144 and subsequent formation of biofilm, which, in turn, can play a major role in the  
145 persistence and transmission of these pathogens. For example, in coastal environments,  
146 *Vibrio cholerae* cells have been shown to survive year round, mostly in a non culturable  
147 state, within clusters of biofilm (Huq *et al.*, 2008). An obvious mechanism by which

148 pathogens in biofilms cause disease is by the seeding and dispersal of a large number of  
149 cells which subsequently can initiate an infection (Huq *et al.*, 2008).

150 Most of the above information on the aquatic ecology of vibrios is coming from human  
151 pathogenic species, particularly the model microorganism and etiological agent of  
152 cholera in humans, *V. cholerae*. Given the substantial lack of knowledge on life outside  
153 the host for the bivalve pathogens *V. splendidus* and *V. aestuarianus*, relevant to the  
154 FP7-European project BIVALIFE (“Controlling infectious diseases in oysters and  
155 mussels in Europe”), the objective of this study was to extensively investigate and  
156 provide solid background information on the ecology of these species, including  
157 occurrence, persistence and survival strategies, in the aquatic brackish environment.

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## 159 **Results and Discussion**

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### 161 **Persistence of *V. splendidus* and *V. aestuarianus* in seawater and sediments under** 162 **different conditions**

163 Seawater temperature and salinity are known to play a pivotal role in affecting the  
164 occurrence and persistence of vibrios in the aquatic environment. The optimal  
165 temperature for growth of *V. splendidus* LGP32 and *V. aestuarianus* 01/32 strains  
166 cultured under laboratory conditions ranged from 20°C to 25°C, and the optimal salinity  
167 was around 20‰. Generation time assessed by culturing the bacteria in Zobell Marine  
168 Broth at 20°C was close to 60 min for both strains.

169 Laboratory microcosm experiments were conducted to assess the persistence (evaluated  
170 in terms of maintenance of culturability and viability over time) of *V. splendidus* LGP32  
171 and *V. aestuarianus* 01/32 strains in both seawater and sediments under experimental  
172 conditions mimicking those found in coastal waters (5°C and 25°C temperature, 20‰  
173 and 35‰ salinity, as usually recorded in such environments over the seasonal cycle).  
174 Following these settings, it was observed that *V. splendidus* LGP32 strain generally  
175 exhibited higher persistence in both seawater and sediments than *V. aestuarianus* 01/32.  
176 In seawater, the majority of *V. splendidus* bacteria maintained viability over a period of  
177 25 days at 5°C, whilst at 25°C a decrease in viability was observed after 10 days  
178 incubation (**Fig. 1a,b**). The different response at the two temperatures may likely be  
179 related to an imbalance between the metabolic rate (higher at 25°C than at 5°C) and  
180 nutrient availability. *V. splendidus* bacteria generally ~~lost~~ culturability in seawater

181 after a short incubation time (less than 5 days) in all tested conditions. After this time,  
 182 ~~the~~ bacterial appeared bright green, very small and coccoid in shape when viewed by  
 183 fluorescence-based Live/Dead assay ~~with~~~~under~~~~an~~ epifluorescence microscopy thus  
 184 likely entering a VBNC physiological state. Unlike *V. splendidus* LGP32, *V.*  
 185 *aestuarianus* 01/32 strain ~~lost~~~~osed~~ both viability and culturability in seawater within 5  
 186 days incubation in almost all experimental settings investigated in this study (**Fig. 1**).  
 187 This suggests that *V. aestuarianus* 01/32 is possibly more demanding in terms of living  
 188 conditions than *V. splendidus* LGP32. Such conditions are probably not met by  
 189 simplified laboratory settings whilst they might be possibly satisfied in the more  
 190 complex natural environment (*e.g.* by the presence of nutrients). According to this,  
 191 initial addition of nutrients (40 mg/L Peptone) to experimental microcosms kept at 25°C  
 192 and 20‰ salinity prolonged the maintenance of viability and cultivability of both strains  
 193 (**Fig. 1e**).

194 In the sediment, results on persistence and survival of the two *Vibrio* species resembled  
 195 those observed in seawater. However, in this compartment, the level of viability (*V.*  
 196 *aestuarianus*) and culturability (*V. splendidus* and *V. aestuarianus*) was generally  
 197 higher throughout the entire duration of the experiment and never dropped below 10<sup>4</sup>  
 198 cells/ml. At least in static conditions, the sediment thus represents a more suitable  
 199 matrix for the persistence of *V. splendidus* LGP32 and *V. aestuarianus* 01/32 in the  
 200 aquatic environment (**Fig. 2**). Accordingly Johnson *et al.*, 2010 observed a protective  
 201 effect in sediment, compared to oyster and water, for *V. parahaemolyticus* and *V.*  
 202 *vulnificus*. This is also probably linked to the fact that sediment provides biotic and  
 203 abiotic surfaces useful for bacterial biofilm development; moreover ~~and~~ concentration  
 204 of organic matter in this compartment is higher than in the overlying water column  
 205 (10.000 to 100.000-fold higher in natural conditions, Vezzulli *et al.*, 2009).

### 207 **Survival strategies in the aquatic environment: adhesion to environmental chitin** 208 **surfaces**

209 The mechanisms promoting *V. splendidus* and *V. aestuarianus* persistence in the  
 210 aquatic environment is an issue deserving investigation since it may be crucial for  
 211 understanding the occurrence, distribution and survival of these bacteria and the  
 212 epidemiology of the bivalve ~~morbidity~~~~sufferance~~ and mortality. To this aim, the  
 213 capability of *V. splendidus* LGP32 and *V. aestuarianus* 01/32 strains to interact with

214 both chitin particles and plankton crustaceans (*Tigriopus fulvus* copepods) was analyzed  
215 *in vitro*. Since *V. cholerae* adhesion to chitin substrates ~~has been~~ extensively  
216 investigated and the main related mechanisms have been well characterized (Pruzzo *et*  
217 *al.*, 2008), *V. cholerae* O1 El Tor 14034 attachment to both substrates was also assessed  
218 as a reference and used for comparison. Adhesion assays showed that *V. splendidus* and  
219 *V. aestuarianus* strains attached to both tested substrates. *V. splendidus* LGP32 showed  
220 a higher efficiency in attachment to chitin particles and copepods than *V. aestuarianus*  
221 01/32 strain (T-test,  $p < 0.05$ ) (**Table 1**). However, both strains were less adhesive than  
222 *V. cholerae* 14034 toward the tested substrates (T-test,  $p < 0.05$ ) (**Table 1**).

223 In order to preliminary assess the nature of the ligands mediating LGP32 and 01/32  
224 attachment to environmental chitin surfaces, the presence of genes encoding for known  
225 chitin binding adhesins was studied. In particular, we analyzed the presence of two  
226 ligands mediating the attachment of *V. cholerae* to environmental chitin surfaces: the  
227 mannose-sensitive haemagglutinin (MSHA) pilus and the N-acetylglucosamine  
228 (GlcNAc)-binding protein A (GbpA). The former is a type 4 pilus mediating binding to  
229 D-mannose-containing receptors and facilitating adhesion to chitin independent of the  
230 surface chemistry (Meibom *et al.*, 2004), whereas the latter is a cell surface protein  
231 which specifically recognizes GlcNAc residues (Stauder *et al.*, 2012). ~~Was a result, we~~  
232 found the *mshA* gene in both strains and the *gbpA* gene only in *V. aestuarianus* 01/32  
233 strain. Experiments of adhesion to chitin and copepods were then performed in the  
234 presence of GlcNAc and D-mannose that in *V. cholerae* act as inhibitors of interactions  
235 with chitin containing substrates mediated by GbpA and MSHA, respectively. As  
236 shown in **Table 1**, in the presence of these sugars, the level of *V. aestuarianus* 01/32  
237 attachment to both chitin particles and copepods was significantly lower than in the  
238 controls (T-test,  $p < 0.05$ ) with percentage of inhibition ranging from 43% to 62%, thus  
239 supporting the role of both ligands in attachment of this strain to chitin containing  
240 substrates. In contrast, neither the addition nor pretreatment of *V. splendidus* LGP32  
241 cells with the sugars reduced the level of bacterial attachment to chitin and copepods.  
242 These results point out to a non significant contribution of MSHA and GlcNAc binding  
243 ligands to LGP32 interactions with chitin substrates. We can speculate that adhesins  
244 binding to GlcNAc oligomers and/or non specific mechanisms, such as those mediated  
245 by hydrophobic forces and electrostatic bonds, are involved in the process. Recently, the  
246 major outer membrane protein OmpU of *V. splendidus* LGP32 was shown to play a role



247 in the attachment and invasion of oyster hemocytes (Duperthuy *et al.*, 2011). However,  
248 to date, the involvement of this protein in the attachment to environmental substrates  
249 (*e.g.* chitin surfaces) is still unknown.

250

### 251 **Survival strategies in the aquatic environment: biofilm formation**

252 Adhesion of *Vibrio* cells to solid substrates such as environmental chitin surfaces can  
253 lead to the formation of biofilm. No information is currently available on the capability  
254 of *V. splendidus* and *V. aestuarianus* to form biofilms; however, studies conducted on  
255 other *Vibrio* species such as *V. cholerae* have clearly shown that biofilm constitutes a  
256 successful survival mechanism increasing the ability of the bacteria to persist under  
257 diverse environmental conditions. (Hall-Stoodley and Stoodley, 2005; Matz *et al.*,  
258 2005). We investigated biofilm formation of *V. splendidus* LGP32 and *V. aestuarianus*  
259 01/32 strains on PVC surfaces at temperatures (18°C and 24°C) relevant in estuarine  
260 and marine environments (**Fig. 3**). As for adhesion assays, *V. cholerae* O1 El Tor 14034  
261 was also tested. For the purpose of comparative analysis, arbitrarily defined categories  
262 were used (see “Experimental procedures”). Such categories classified bacteria as non-  
263 ~~producer~~~~adherent~~, weak ~~producer~~~~ly-adherent~~, and strong ~~producer~~~~ly-adherent~~.

264 At both temperatures, *V. splendidus* LGP32 strain showed a greater capability to form  
265 biofilm on PVC surfaces over the course of the experiment (strongly ~~adherent~~~~producer~~  
266 with average  $OD_{545}=0.40\pm 0.02$  at 18°C and average  $OD_{545}=0.36\pm 0.03$  at 24°C) than *V.*  
267 *aestuarianus* 01/32 strain (non-~~adherent~~~~producer~~ with average  $OD_{545}=0.05\pm 0.04$  at  
268 18°C and average  $OD_{545}=0.02\pm 0.01$  at 24°C). Both strains showed lower capability to  
269 form biofilm than *V. cholerae* 14034 ( $OD_{545}=0.8\pm 0.08$  at 18°C;  $OD_{545}=0.8\pm 0.08$  at  
270 24°C). These results might help to explain higher persistence of *V. splendidus* LGP32  
271 than *V. aestuarianus* 01/32 strain observed in the sediment by laboratory experiments.

272

### 273 **Survival strategies in the aquatic environment: entering into the viable but** 274 **nonculturable (VBNC) state**

275 Since VBNC state in vibrios is commonly induced by temperatures below 10°C (Oliver,  
276 2005), we studied the effect of prolonged exposure (90 days) of *V. splendidus* LGP32  
277 and *V. aestuarianus* 01/32 strains to artificial sea water (ASW 20‰ salinity) at 5 °C  
278 under starving condition.

279 Incubation of bacteria under such conditions resulted in different cell responses by the  
280 two *Vibrio* strains. After 90 days incubation, the number of CFU/ml drastically dropped  
281 to  $10^1$  CFU/ml, corresponding to 0,00001% of total cells, for *V. aestuarianus* 01/32  
282 strain, and  $10^4$  CFU/ml, corresponding to 0,01% of total cells, for *V. splendidus* LGP32  
283 (Fig. 4). Consistently with results from laboratory microcosm experiments (Fig. 1),  
284 46,8% of *V. splendidus* LGP32 cells retained viability after incubation (as determined  
285 by fluorescence-based Live/Dead assay for cell viability), whilst the majority of *V.*  
286 *aestuarianus* 01/32 cells (94%) appeared dead after prolonged exposure to low  
287 temperature in ASW (20‰ salinity) (Fig. 4).

288 In order to detect the presence of VBNC cells, the presence and relative abundance  
289 (expressed as percentage of total cells) of viable and culturable bacteria was also  
290 investigated on different cell size fractions ( $> 0.2\mu\text{m}$  and  $< 0,2\mu\text{m}$ ) obtained by filtering  
291 the bacterial suspensions through polycarbonate filters of  $0,2\ \mu\text{m}$  pore size. Only  
292 fractions of *V. splendidus* and *V. aestuarianus* containing bacterial cells larger than  $0.2$   
293  $\mu\text{m}$  yielded colonies on culture media (Fig. 4). In contrast, for both strains, cells smaller  
294 than  $0.2\ \mu\text{m}$  completely lost culturability ( $<0.1$  CFU/ml) (Fig. 4). Retention of  
295 membrane integrity of this cell fraction was investigated using the fluorescence-based  
296 Live/Dead assay for cell viability. It was shown that the majority of the cells ( $>70\%$ )  
297 indeed retained viability. Contamination was ruled out by testing DNA extracted from  
298 *V. splendidus* and *V. aestuarianus* bacteria smaller than  $0.2\ \mu\text{m}$  with specific primers.  
299 Interestingly, VBNC bacteria maintained the same virulence related genes found in the  
300 parents (*mshA*, *ompU* and metalloprotease genes for *V. splendidus* LGP32 and *mshA*,  
301 *gbpA*, *ompU* and metalloprotease genes for *V. aestuarianus* 01/32). In addition,  
302 observation by both standard and epifluorescence microscopy showed that bacteria  
303 besides becoming smaller in size changed from rod to ovoid or coccoid morphology  
304 (Fig. 5).

305 Overall, these results provide evidence that *V. splendidus* LGP32 and, to a lesser extent,  
306 *V. aestuarianus* 01/32 are capable of entering a VBNC physiological state after  
307 extended incubation at  $5^\circ\text{C}$ , a condition that could be met during the winter season in  
308 shallow brackish environment where bivalve farming generally takes place. Besides  
309 contributing to the bacterial persistence in such environments, bacterial cells in the  
310 VBNC state might also retain virulence properties and might re-activate under favorable  
311 conditions into an actively metabolizing and culturable form, as shown in earlier studies

312 for other *Vibrio* species (Pruzzo *et al.*, 2003). Studies in this direction are currently  
313 undergoing in our laboratory.

314

### 315 **Occurrence, temporal variations and environmental reservoirs of *V. splendidus* and** 316 ***V. aestuarianus* in the Goro Lagoon (Northern Adriatic Sea, Italy)**

317 In order to complement and validate the results obtained by laboratory experiments, the  
318 occurrence and temporal variations of *V. aestuarianus* and *V. splendidus*-clade bacteria  
319 were investigated in the Goro Lagoon (Northern Adriatic Sea, Italy) (**Fig. 6**) in different  
320 environmental matrices (seawater, sediment, zooplankton) from May 2011 to January  
321 2013. Identification and enumeration of these bacteria were conducted in parallel in  
322 both mussels (*Mytilus galloprovincialis*) and oysters (*C. gigas*) collected at the same  
323 location. In addition, the concentration of *Vibrio* spp. bacteria was also assessed in order  
324 to evaluate the general ecological features of the *Vibrio* community in the study area.

325 As shown in **Fig. 7**, the number of total and culturable *Vibrio* spp. bacteria did not show  
326 a significant temporal trends in environmental matrices and bivalves. Interestingly, high  
327 number of culturable *Vibrio* spp. were found also in winter months (February 2012 and  
328 January 2013 SST<5°C) in contrast to what it is commonly observed in temperate  
329 coastal environment where vibrios can be generally cultured only during the warm  
330 season ([Johnson \*et al.\*, 2010](#); [Oliver \*et al.\*, 2013](#)). No significant correlation was found  
331 between SST and *Vibrio* spp. concentration in all environmental matrices with the  
332 exception of zooplankton where a significant positive correlation with temperature was  
333 observed (Pearson correlation analysis,  $p<0.05$ ) (**Supplementary Fig. 1**). The absence  
334 of a clear temporal trend in *Vibrio* spp. abundance as well as the presence of culturable  
335 vibrios in seawater during cold months in the Goro Lagoon could be related to the high  
336 nutrients content of these waters (Viaroli *et al.*, 2006). This condition is known to favor  
337 bacterial persistence and maintenance of culturability in *Vibrio* species (Oliver, 2005).  
338 Nevertheless, the number of *Vibrio* spp. determined by Real-Time PCR was at least one  
339 order of magnitude greater than culturable counts also suggesting the presence of a large  
340 fraction of dead and/or VBNC bacterial cells in the studied environment (**Fig. 7**).

341 *V. aestuarianus* and *V. splendidus*-clade bacteria were found in the lagoon by Real Time  
342 PCR studies in association with a number of environmental matrices and living  
343 organisms that may function as reservoirs and/or hosts. In particular, higher  
344 concentrations of *V. splendidus*-clade bacteria were associated to zooplankton compared

345 to the other environmental matrices, with values generally exceeding  $10^6$  cells/g both in  
346 warm and cold months (**Fig. 8**). These bacteria were also frequently detected in oysters  
347 and mussels; when present, their concentration ranged from  $1.1 \times 10^5$  to  $1.5 \times 10^7$  cells/g  
348 and from  $2.8 \times 10^2$  to  $3.8 \times 10^7$  cells/g for oyster and mussel respectively.

349 In seawater and sediment compartments, when present, their concentration ranged from  
350  $1.9 \times 10^1$  to  $7.6 \times 10^2$  cells/ml and from  $7.4 \times 10^2$  to  $1.6 \times 10^5$  cells/g, respectively. The  
351 sediment was the compartment where *V. splendidus*-clade bacteria were more  
352 frequently found during the study period (9 out of 11 sampling dates) and may thus  
353 represent a suitable niche for their persistence in the environment. *V. splendidus*-clade  
354 bacteria were also found associated to a variety of other environmental substrates  
355 commonly found in the lagoon including large benthic crustaceans (*Dyspanopeus sayi*,  
356 *Upogebia pusilla*, *Cratigon* sp., *Carcinus aestuarii*), *Gracilaria verrucosa* macroalgae  
357 and *Tapes philippinarum* clam (data not shown).

358 In contrast to *V. splendidus*, *V. aestuarianus* cells were rarely detected in the Goro  
359 lagoon samples. In particular, high concentrations of *V. aestuarianus* bacteria were  
360 found associated to zooplankton during warm months, with values ranging from  $1.4 \times 10^5$   
361 to  $6.9 \times 10^6$  cells/g, and only sporadically found in mussels, oysters, seawater and  
362 sediments (**Fig. 8**). In particular, during cold months, *V. aestuarianus* was detected only  
363 once (January 2013) in the sediment compartment. The sediment may thus represent an  
364 environmental reservoir also for this species where, in accordance with results from  
365 laboratory experiments (**Fig. 2**), the bacteria can find a favorable environment for  
366 overwintering (Vezzulli *et al.*, 2009).

367 Interestingly data on the survival of *V. splendidus* LGP32 and *V. aestuarianus* 01/32 in  
368 *C. gigas* and *M. galloprovincialis* showed that both strains are resistant to killing by  
369 oyster hemolymph (serum + hemocytes) while *V. aestuarianus* only is sensitive to  
370 killing by *M. galloprovincialis* hemocytes (Balbi *et al.*, 2013).

371 Culture based analyses showed that *V. splendidus*-clade bacteria are often present in  
372 culturable form in the different matrices. Sequencing of *pyrH* gene of these isolates  
373 showed that half of them belonged to the *V. splendidus* species (2 from oysters, 2 from  
374 mussels, 2 from zooplankton, 4 from sediment and 4 from seawater) suggesting that  
375 bacteria belonging to *V. splendidus* species, as well as those belonging to *V. splendidus*  
376 clade, are widespread in the Goro lagoon and can maintain culturability also during cold  
377 months (**Supplementary Table 1**). Regarding culturable *V. aestuarianus* bacteria, they

378 were found only sporadically in warm months. A total of 12 strains was isolated, 3 from  
379 oysters, 3 from mussels, 5 from zooplankton and 1 from sediment. These results,  
380 besides being consistent with those obtained by Real-Time PCR, also support what  
381 observed in laboratory microcosms, *i.e.* higher capability to persist in the environment  
382 showed by *V. splendidus* in comparison with *V. aestuarianus*, which rapidly loses  
383 culturability in all tested conditions.

384 Overall, environmental variables such as SST and salinity do not seem to have a  
385 significant influence on the presence and concentration of these bacteria in  
386 environmental matrices and bivalves (**Supplementary Fig. 1, 2**). In agreement to what  
387 we observed for the whole *Vibrio* spp. community, this may be related to the ecological  
388 features of the Goro lagoon; in fact it is well known that in shallow coastal eutrophic  
389 environment microbial abundance might lack a clear seasonal trend being marginally  
390 subjected to the influence of the main environmental variables (Vezzulli and Fabiano,  
391 2006; Moreno *et al.*, 2008, 2011). Indeed, a significant positive correlation was only  
392 found between SST and *V. splendidus*-clade/*V. aestuarianus* bacteria associated to  
393 zooplankton (Pearson correlation analysis,  $p < 0.05$ ), a condition which has already been  
394 described in other *Vibrio* species such as *V. cholerae* (Stauder *et al.*, 2010)  
395 (**Supplementary Fig. 1**). Surprisingly, a significant negative correlation was found  
396 between SST and *V. splendidus*-clade bacteria in seawater (Pearson correlation analysis,  
397  $p < 0.05$ ). A full explanation for this is not available, however this result is consistent to  
398 observations made in other oyster farming sites across Europe (Chris Roger, personal  
399 communication).

400

#### 401 **Occurrence of genes linked to environmental persistence and virulence in *V.*** 402 ***splendidus* and *V. aestuarianus* isolates from the Goro lagoon**

403 To evaluate the presence and distribution of genes linked to environmental persistence  
404 and virulence in environmental isolates from the Goro lagoon, genes encoding for  
405 MSHA, GbpA, OmpU and zinc metalloprotease were evaluated in 39 strains including  
406 12 *V. aestuarianus* and 14 *V. splendidus* isolates. The remaining isolates belonged to  
407 other species of the *V. splendidus* clade (**Supplementary Table 1**).

408 Regarding genes encoding for adhesins involved in attachment to chitin, all *V.*  
409 *splendidus* isolates lacked both *mshA* and *gbpA*. In contrast, almost all *V. aestuarianus*  
410 strains (10 out of 12) were positive for *gbpA* and three strains carried the *mshA* gene

411 (Fig. 9). These results suggest that adhesion ligands, mainly GbpA, are diffused among  
412 lagoon populations of *V. aestuarianus* and their expression might contribute to the  
413 persistence of these bacteria in the environment through chitin surface colonization. The  
414 fact that they are not found among *V. splendidus* species supports the possible role of  
415 other unknown ligands in colonization of environmental substrates.

416 As regards to genes associated to virulence (Le Roux *et al.*, 2007; Labreuche *et al.*,  
417 2010; Duperthuy *et al.*, 2011), most *V. splendidus* strains were observed to carry the  
418 *ompU* gene (11 out of 14) whilst the zinc metalloprotease gene was detected in half of  
419 the isolates (7 out of 14). In the case of *V. aestuarianus*, the majority of isolates was  
420 positive for OmpU (10 out of 12) and zinc metalloprotease (11 out of 12) genes (Fig. 9,  
421 Supplementary Table 1). Interestingly, genes encoding OmpU protein and zinc  
422 metalloprotease were also detected in isolates belonging to other species closely related  
423 to *V. splendidus* including *V. celticus*, *V. crassostrea*, *V. chagasii* and *V. lentus* (Fig. 9,  
424 Supplementary Table 1).

425 These data suggest that virulence-related genes are widely diffused in the environment  
426 not only in the species potentially pathogenic for oysters but also in other harmless  
427 bacterial species. Such strains might thus represent a reservoir of these genes in the  
428 aquatic environment that might be exchanged among *Vibrio* strains by horizontal gene  
429 transfer (HGT) (Sechi *et al.*, 2000).

430

### 431 Concluding remarks

432 Reducing the impact of environmental pathogens mostly relies on knowledge of their  
433 biology and ecology including the mechanisms that allow their survival outside the host.  
434 Marine bivalves such as oysters are typically reared in shallow brackish waters  
435 frequently subjected to fluctuations of environmental factors such as temperature,  
436 salinity and nutrient concentration. Using both *in vitro* and in field studies, we provided  
437 evidence that the oyster pathogens *V. splendidus* and *V. aestuarianus* persist in the  
438 aquatic environment with different efficiency by adopting various survival strategies,  
439 including adhesion to environmental chitin substrates and biofilm formation.  
440 Zooplankton and the sediment compartment seem to represent important environmental  
441 reservoirs where these bacteria can persist in high abundance and/or can be found  
442 during cold and unfavorable environmental conditions. Both *V. splendidus* and *V.*  
443 *aestuarianus* are also capable of entering a VBNC state after extended incubation at

444 5°C, a condition commonly found during cold season in the aquatic brackish  
445 environment. Interestingly, it was observed that a significant fraction of VBNC cells  
446 showed a reduced size (<0.2 µm) which may have important implication for their  
447 detection in the environment (e.g., the use of standard membranes might miss this  
448 fraction and this should be taken into account in microbiological analysis). Overall,  
449 these data provide new background information on the mechanisms promoting *V.*  
450 *splendidus* and *V. aestuarianus* persistence in coastal water thus contributing to a better  
451 understanding of the epidemiology of their associated diseases.

452

## 453 **Experimental procedures**

454

### 455 ***In vitro* studies**

#### 456 ***Bacterial strains and culture conditions***

457 *V. splendidus* LGP32 and *V. aestuarianus* 01/32 strains kindly provided by Tristan  
458 Renault (IFREMER, La Tremblade, France) were used in this study for laboratory  
459 experiments. Both strains were cultivated aerobically for 24-48 h at 20°C in  
460 Zobell Marine Broth 2216 (Difco Laboratories Inc.). *V. splendidus* LGP32 was also  
461 cultured in Luria–Bertani (LB) agar (Conda laboratories, Madrid, Spain) containing  
462 3% NaCl and *V. aestuarianus* 01/32 in Marine agar 2216 (Difco Laboratories Inc.). *V.*  
463 *cholerae* O1 biovar El Tor strain ATCC 14034 was also used in some experiments and  
464 cultured in LB agar and broth, or Zobell Marine Broth 2216. ASW was obtained by  
465 reconstituting Sea Salts (Sigma–Aldrich, Milan, Italy) with demineralized hypo-osmolar  
466 water to 20‰ and 35‰ final concentration and then autoclaving the resulting solution.  
467 If not differently specified, with the acronym ASW we refer to 35‰ salinity condition  
468 throughout the manuscript.

469

#### 470 ***Setting of experimental microcosms for “survival” and “VBNC induction”***

##### 471 ***experiments***

472 Microcosm vessels for survival experiments of *V. splendidus* LGP32 and *V.*  
473 *aestuarianus* 01/32 strains in seawater were prepared using sterile flasks added with 500  
474 ml of ASW and kept under aseptic conditions at different temperatures and salinity  
475 (25°C, 20‰ salinity; 25°C, 35‰ salinity; 5°C, 20‰ salinity; 5°C, 35‰ salinity). Some  
476 experiments were also performed in the presence of 40 mg/L Peptone (Sigma–Aldrich,

477 Milan, Italy). For survival experiments in the sediment, additional flasks were added  
478 with 10 g of autoclaved sterile sediment collected in the Goro lagoon (see below) and  
479 kept at 5°C under aseptic conditions. The sediment layer was gently covered with ASW  
480 and vessels were let to settle for 24 h before starting the experiments.

481 Single colonies of bacterial strains were picked from agar plates, transferred to 100 ml  
482 of Zobell Marine Broth 2216, and incubated at 20°C. Bacterial cells were harvested by  
483 centrifugation at 4500 x g for 10 min. After centrifugation, the cells were washed twice  
484 with ASW and suspended in the microcosm flasks, prepared as described above at a  
485 final concentration of 10<sup>7</sup> cells/ml(g), as determined by total epifluorescence counts  
486 (Hobbie *et al.*, 1977). Three replicate flasks were prepared for each strain and condition.  
487 Flasks were then regularly monitored for culturability and viability at day 0, 5, 10, 15  
488 and 20 (see methods described on the below).

489 For VBNC induction experiments, additional ASW (20‰ salinity) microcosm flasks  
490 (three replicate flasks for each strain) were inoculated as described above and were  
491 maintained at 5°C, a condition know to induce the VBNC state in other *Vibrio* species  
492 (Oliver *et al.*, 2010). After 3 months, the total, culturable and viable cells present in  
493 each microcosm were determined (see methods described on the below). Bacterial cells  
494 were then divided into two size fractions (>0.2 µm and <0.2µm) by passages through a  
495 0.2 µm nucleopore filter (Millipore, Milan, Italy). Total, culturable and viable cells of  
496 each size fraction were also evaluated.

497

#### 498 ***Nucleic acid extraction***

499 DNA from bacterial cultures and microcosm water samples was extracted with the High  
500 Pure Polymerase Chain Reaction (PCR) Template Preparation Kit (Roche Diagnostics,  
501 Mannheim, Germany) according to the manufacturer's instructions. Nucleic acids from  
502 sediment samples were extracted with the UltraClean soil DNA kit (MoBio  
503 Laboratories, Solana Beach, CA, USA) according to the manufacturer's instructions and  
504 recommendation provided for the use with marine sediment (Luna *et al.*, 2006). The  
505 amount of extracted DNA was quantified using the Quantifluor double-stranded DNA  
506 quantification kit (Promega Italia, Milan, Italy).

507

#### 508 ***Propidium monoazide (PMA) treatment of bacterial cells in microcosm samples***



509 Propidium monoazide (PMA) (Gentaur molecular product, Milan, Italy) was dissolved  
 510 in 20% dimethyl sulfoxide to create a stock concentration of 20 mM and stored at -20°C  
 511 in the dark. A first set of trial experiments was conducted for setting optimal protocols  
 512 to be employed in the study using variable concentrations of PMA and light exposure  
 513 times.

514 According to the results, 500 µl of seawater and sediment suspensions (1 g of sediment  
 515 were resuspended in 5 ml ASW, vigorously vortexed for 30 s and treated in a sonication  
 516 bath for 60 s for a total of three times) were treated with PMA to final concentrations of  
 517 50 µM and 100 µM for water and sediment, respectively. Following 5 min incubation in  
 518 the dark with occasional mixing, samples were light exposed for 2 min using a 650W  
 519 (3200K) halogen light source (Uniquartz, FIEF Lighting, Ferrara, Italy). The sample  
 520 tubes were placed about 20 cm from the light source and were laid horizontally with  
 521 gentle shaking on ice to avoid excessive heating. After photo-induced cross-linking,  
 522 cells were pelleted at 4.500 x g for 10 min prior to DNA isolation as previously  
 523 described.

524

#### 525 ***Enumeration of total and viable bacterial cells by Real-Time PCR***

526 Real-time PCR for the enumeration of *V. splendidus* LGP32 and *V. aestuarianus* 01/32  
 527 in seawater and sediment samples from experimental microcosms were performed using  
 528 a Taqman Real-Time PCR protocol with the LightCycler (Roche Diagnostics, Mannheim,  
 529 Germany). *V. splendidus* clade (16SspF2 ATCATGGCTCAGATTGAACG; 16SspR2  
 530 CAATGGTTATCCCCACATC; 16S probe FAM-  
 531 CCCATTAACGCACCCGAAGGATTG-BHQ1; IFREMER, 2013) and *V. aestuarianus*  
 532 (DNAj F GTATGAAATTTTAAGTACCCACAA; DNAjR  
 533 CAATTTCTTTTGAACAACCAC; DNAj probe FAM-  
 534 TGGTAGCGCAGACTTCGGCGAC – BHQ2; IFREMER, 2013) specific primers and  
 535 probe were used in the assays. Briefly, each reaction mixture contained 1X LighCycler  
 536 Taqman master (Roche Diagnostics, Mannheim, Germany) and 1 µM of each primer  
 537 and 0.1 µM of each probe in a final volume of 20 µl. The PCR program used was as  
 538 follows: initial denaturation at 95°C for 10 min, subsequent 45 cycles of denaturation at  
 539 95°C for 10 s, annealing at 60°C for 15 s and elongation at 72°C for 1 s, followed by  
 540 final elongation at 72°C for 10 min. Five µl of DNA template (DNA concentration for  
 541 all samples varied from 1 to 10 µg/ml) were added to the reaction mixture. Accurately

542 quantified copy number genomic DNA of *V. splendidus* LGP32 and *V. aestuarianus*  
543 01/32 strains was used as a standard. For quantification, the log of the number of  
544 genome units (GI) of a dilution series of the sample was plotted *versus* the cycle number  
545 at which the fluorescent signal increased above background or threshold (Ct value).  
546 This approach was used because, for detection and enumeration of bacterial cells in  
547 environmental samples, it is easier to understand results in actual cell numbers than in  
548 DNA concentrations or gene copy numbers. If necessary, a correction factor was  
549 applied to account for over-/under-estimation due to the effect of different efficiencies  
550 between the standard and sediment samples.

551

#### 552 ***Enumeration of total and viable bacterial cells by Epifluorescence microscopy***

553 Total (TBN) and viable (VBN) bacterial cells were enumerated in seawater samples  
554 from VBNC induction experiments using epifluorescence microscopy. TBN were  
555 assessed using the Acridine Orange Direct Count technique of Hobbie *et al.* (1977)  
556 whilst VBN were evaluated using the Live/Dead BacLight Bacterial Viability kit for  
557 microscopy and quantitative assays (Molecular Probes, Eugene, OR) following the  
558 manufacturer's instructions. Fluorescence from the stained cells was viewed using an  
559 epifluorescence microscope equipped with filter set 9 (Excitation 450-490; Beam  
560 Splitter FT510, Emission LP520) and filter set 43 (Excitation 545-25; Beam Splitter  
561 FT570, Emission BP605/70) (Zeiss Universal Microscope).

562

#### 563 ***Enumeration of culturable bacterial cells***

564 To enumerate culturable bacteria in samples from experimental microcosms, 200 µl of  
565 seawater and sediment suspension (1 g of sediment was resuspended in 5 ml ASW,  
566 vigorously vortexed for 30 s and treated in a sonication bath for 60 s for a total of three  
567 times) were serially diluted ( $10^{-1}$  to  $10^{-8}$ ) with ASW and spread in triplicate onto  
568 appropriate culture media. Plates were incubated at 20°C for 48 h and bacterial colonies  
569 were counted. Results were expressed as CFU/ml(g) (colony-forming units per  
570 milliliter).

571

#### 572 ***Adhesion assays***

573 To assess bacterial attachment to chitin particles one volume bacterial suspension  
574 ( $5 \times 10^7$  cell/ml) was added to UV-sterilized chitin purified from crab shell (2.5 mg;

575 Sigma Chemical Co., St Louis, MO, USA) and the mixture was incubated for 1 h at  
576 20°C with shaking. A sample without chitin was also prepared as a control. After  
577 incubation, chitin particles were repeatedly washed (x3) with 1 ml ASW to remove  
578 unattached bacteria. 200 µl of PCR grade water was added to chitin pellets and DNA  
579 was extracted by boiling. The number of attached bacteria was determined by Real  
580 Time PCR using protocols described on the above. In addition, to preliminary define the  
581 nature of bacterial ligands involved in interactions with chitin, adhesion assays were  
582 also performed by either pretreating bacteria with or in the presence of sugars (GlcNAc  
583 and D-mannose) at the final concentration of 10 mg/ml (1h incubation at room  
584 temperature with shaking).

585 To assess bacterial attachment to copepods a similar protocol was followed. *T. fulvus*  
586 copepods belonging to the *Harpacticoida* family were cultured in filter-sterilized ASW  
587 and fed with unicellular phytoplanktonic algae (*Tetraselmis suecica*). Before the  
588 association assay, the copepods were vigorously washed 10 times with ASW to remove  
589 most adhering bacteria and heat killed (65°C per 15 min). One ml of bacterial  
590 suspension ( $1 \times 10^8$  cell/ml) was then added to 10 washed copepods in a tube and  
591 incubated at room temperature over night. After overnight incubation, copepods were  
592 collected and gently washed three times with phosphate buffered saline (PBS) solution  
593 (137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) PBS to remove non-adherent  
594 bacteria. 200 ul of PBS was then added to the washed copepods and DNA was extracted  
595 as previously described. The number of attached bacteria were determined by Real  
596 Time PCR. All adhesion assays were performed in triplicate.

597

### 598 **Biofilm assays**

599 For biofilm formation analysis, the microtiter plate method (Stepanovic *et al.*, 2000)  
600 was followed with few modifications. Overnight bacterial cultures grown in  
601 ZobellMarine Broth 2216 were diluted to  $Ab_{S_{640}} = 0.02$ . Sterilized microtitre plates (96  
602 wells) made of PVC were previously conditioned with 200 µl ASW (overnight  
603 incubation at room temperature). Each well was filled with 180 µl Zobell medium and  
604 20 µl of the cell suspension and incubated for 24, 48, 72 h and 168 h at 18°C and 24°C.  
605 Biofilm was gently washed to eliminate non-adherent bacteria and stained with 0.01%  
606 crystal violet (100 µL). After 15 min incubation at room temperature, wells were  
607 gently washed with water, and bound dye was extracted from stained cells by adding

608 200  $\mu$ l of ethanol : acetone (8:2) per well. After about 1 h incubation, biofilm formation  
609 was quantified by measuring the absorbance (Abs) of the solution at 5450 nm.  
610 For the purposes of comparative analysis, we classified bacteria into three arbitrarily  
611 defined categories based upon Abs<sub>5450</sub> of the supernatant as follow: Abs<sub>5450</sub>  $\leq$  0.150 =  
612 non-~~producer~~~~adherent~~ bacteria; Abs<sub>5450</sub>  $\geq$  0.250 = strongly ~~adherent~~ ~~producer~~;  
613 0.150 < Abs<sub>5450</sub> < 0.250 = weak ~~producer~~ ~~ly-adherent~~. Tests were done in triplicate on three  
614 separate occasions and the results averaged.

615

## 616 In field studies

### 617 *Experimental design and field sampling*

618 Studies in the field were carried out in the Sacca di Goro lagoon (Northern Adriatic Sea,  
619 Italy). The lagoon at the south of the Po River Delta is among the most important  
620 shellfish aquaculture systems in Italy and it has been extensively studied by previous  
621 investigators (Viaroli *et al.*, 2006). The total surface area is 830 km<sup>2</sup> for the watershed  
622 and 26 km<sup>2</sup> for the lagoon, respectively. The watershed is exploited for agriculture,  
623 whilst the coastal lagoon is one of the most important European sites for bivalve  
624 farming. The lagoon is a sheltered and shallow area (less than 5 m average depth) and it  
625 is characterized by a low circulation regime and temperatures ranging from 4°C in  
626 winter to 30°C in summer.

627 An experimental station within the FP7-European project BIVALIFE was established in  
628 the inner part of the lagoon for the cultivation of mussel *M. galloprovincialis* and oyster  
629 *C. gigas* (Fig. 6).

630 In 2011 commercial French seed of *C. gigas* triploids about 6 month old and indigenous  
631 diploid *M. galloprovincialis* of similar size (>600 individuals per species) were placed  
632 in alternate sectors of *Ostrea*<sup>tm</sup> baskets (20 for each species) which were subsequently  
633 piled and suspended in shallow water (0.5-1 m depth) of the Goro lagoon. The baskets  
634 were regularly monitored to avoid fouling and overfilling due to the bivalve growth.  
635 After about one month of acclimation, the cohabiting oysters and mussels were sampled  
636 at low tide. The same work plan was followed in 2012 with the exception that diploid *C.*  
637 *gigas* spat was obtained from a local hatchery (Chioggia, Venice).

638 Eleven sampling campaigns were carried out on May 30th 2011, June 20th 2011, July  
639 18th 2011, August 1st 2011, October 10th 2011, February 14th 2012, May 21st 2012,  
640 June 18th 2012, July 16th 2012, September 3rd 2012, January 14th 2013. During each

641 campaign water, sediment, zooplankton and bivalve (*M. galloprovincialis* and *C. gigas*)  
642 samples were collected. Water samples were collected into sterile bottles and filtered on  
643 a piece of the 200- $\mu\text{m}$  net in order to remove the highly particulate matter (zooplankton  
644 included). Five hundred milliliters of the resulting water were then filtered onto a 0.22-  
645  $\mu\text{m}$ -pore-size Millipore membrane (47 mm in diameter) (Millipore, Milan, Italy). Filter  
646 bound material was suspended in ASW (50 ml) by vortexing and used for  
647 microbiological and molecular investigations. Surface sediment samples (0-2 cm) were  
648 collected by manual sediment core. For DNA extraction and direct bacterial  
649 quantification by Real-Time PCR, 1 g of sediment was used. For culture dependent  
650 assays, sediment aliquots (5 g) were suspended in 50 ml ASW, vigorously vortexed for  
651 30 s and treated in a sonication bath for 60 s (for a total of three times); supernatants  
652 were collected and processed. Zooplankton organisms ( $>200 \mu\text{m}$ ) were collected by  
653 dragging the water horizontally, at a depth of about 1 m, with a 200- $\mu\text{m}$ -mesh plankton  
654 net. Five to 10  $\text{m}^3$  of water were dragged at each sampling. In the laboratory, the whole  
655 zooplankton suspension was passed through a 200  $\mu\text{m}$  mesh filter and total zooplankton  
656 was collected; after washing with ASW, 0.25 g of zooplankton were used for DNA  
657 extraction and bacterial quantification by Real-Time PCR. Another 1 g aliquot was  
658 suspended in ASW (50 ml), vigorously vortexed for 30 s and treated in a sonication  
659 bath for 60 s (for a total of three times) and used for culture dependent assays.

660

661 ***Detection, enumeration and isolation of culturable vibrios in environmental and***  
662 ***bivalve samples***

663 The most-probable-number technique was used to enumerate the number of culturable  
664 *Vibrio* spp., in the water, zooplankton and sediment samples. Three dilution series  
665 (1:10) were carried out in five replicate culture tubes, starting with 0.5 ml of prepared  
666 samples and 4.5 ml of Alkaline Peptone Water (APW, final pH  $8.6 \pm 0.2$ ) as the growth  
667 medium. Tubes were incubated at 24°C for 8 h in the dark and growth was assessed by  
668 plating each APW tube onto Thiosulfate Citrate Bile Sucrose (TCBS) (Conda  
669 laboratories, Madrid, Spain) plates. Plates were incubated at 20°C for 48 to 96h. For  
670 enumeration of *Vibrio* spp. in oysters and mussels, pools of 30 animals were  
671 homogenized in a Polytron PT 3000 Kinematica AG homogenizer and dilutions to the  
672 1/100 and 1/10.000 were performed in ASW. One hundred  $\mu\text{l}$  of those dilutions were  
673 plated on TCBS plates and incubated for 48 to 96 h at 20°C. Microorganisms were

674 counted as *Vibrio* spp. if they formed 2-3 mm yellow or green colonies on TCBS  
675 medium, were Gram-negative and curved-bacillus-shaped, oxidase-positive and  
676 fermentative with glucose by the oxidation/fermentation test. For the isolation and  
677 identification of *V. splendidus* clade bacteria and *V. aestuarianus*, colonies were tested  
678 by clade and species-specific Real Time PCR assays respectively, as previously  
679 described. Bacterial isolates identified as belonging to *V. splendidus* clade were further  
680 identified at the species level by sequencing the *pyrH* gene following the protocol  
681 described by Tall *et al.*, (2013).

682

### 683 ***Detection and enumeration of vibrios in environmental and bivalve samples by Real-*** 684 ***Time PCR***

685 Nucleic acids from zooplankton, mussels (0.25 g) and oyster (0.25 g) samples were  
686 extracted with the High Pure Polymerase Chain Reaction (PCR) Template Preparation  
687 Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's  
688 instructions. Nucleic acids from seawater and sediment samples were extracted as  
689 previously described for *in vitro* experiments. For *Vibrio* spp. enumeration genus-  
690 specific primers (F-GGCGTAAAGCGCATGCAGGT; R-  
691 GAAATTCTACCCCCTCTACAG, Thompson *et al.*, 2004) and the LightCycler-  
692 FastStart DNA Master SYBR Green I kit optimised for use with glass capillaries were  
693 used following conditions described in Vezzulli *et al* (2009). For the enumeration of *V.*  
694 *splendidus* clade bacteria and *V. aestuarianus* a Taqman Real-Time PCR protocol was  
695 performed as previously described.

696

### 697 ***Characterization of environmental isolates***

698 All *Vibrio* isolates belonging to *V. splendidus* clade and *V. aestuarianus* were also  
699 analyzed for the presence of genes encoding for adhesins to chitin substrates (*mshA* and  
700 *gbpA*) and genes encoding for virulence associated traits (*ompU* and *vsm/vam*). Primers  
701 used were the following: mshavsF- ACTTACAAGGCGATGCTCGT and mshavsR-  
702 TCCACGGCTTCTTCAATACC (*mshA* of *V. splendidus*, this study); mshavaF-  
703 GCGGTTTCACCTTATTGAA and mshavaR -ATACCAGCGGCACCATCTAA  
704 (*mshA* of *V. aestuarianus*, this study); gbpAvaF-TATTACTCCCGCGCAAATC and  
705 gbpAvaR-ATGCCATAGAGCGGAGAGAA (*gbpA* of *V. aestuarianus*, this study);  
706 gbpAF-TGTCGTTGAAGGAAATATGGTG and gbpA-R

707 TCTTTCAGATAAATCGGGTTGG (*gbpA* of *V. splendidus*-clade, Stauder *et al.*,  
 708 2010); 2494-5 TGACCGTGCTGACAACATGC and 2494-6  
 709 TTAGAAGTCGTAACGTAGACC (*ompU* of *V. splendidus* and *V. aestuarianus*,  
 710 Duperthuy *et al.*, 2010); *vsmF*-TCCAACAGAGCCTCGTCG and *vsmR*-  
 711 AAGGGCGTTCTGGCATAG (*vsm* of *V. splendidus*, Liu *et al.*, 2013); *vamF*-  
 712 TTGACGTGGTTGATGCATTT and *vamR*-AGATCGGCATCACCTGTACC (*vam* of  
 713 *V. aestuarianus*, this study)

714

### 715 ***Environmental variables***

716 Daily records of SST (°C) and salinity values (PSU) were measured in the Goro lagoon  
 717 from May 2011 to January 2013 by means of a CTD-multiprobe.

718

### 719 ***Statistical tests***

720 One-way unpaired T -test was used to compare average values of adhesion and biofilm  
 721 formation among bacterial strains. Pearson correlation analysis was performed to  
 722 investigate the relationship between bacterial and environmental variables.

723

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733

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925 **Table 1**

926 Attachment of *V. splendidus* LGP32, *V. aestuarianus* 01/032 and *V. cholerae* O1 14034  
 927 strains to chitin particles and to the copepod *T. fulvus*. Values are means of three assays  
 928 performed on the same day with the same bacterial suspension. Percentages of  
 929 inhibition in comparison with untreated control are reported in brackets; the symbol –  
 930 means no inhibition, nd means not determined

931

Strain	Experimental conditions	Attachment to chitin (bacteria per 2,5 mg of chitin particles)	Attachment to copepods (bacteria per copepod)
<i>V. aestuarianus</i> 01/032	control	$6.0 \times 10^4 \pm 5 \times 10^3$	$3.3 \times 10^2 \pm 4 \times 10^1$

<i>V. splendidus</i> LGP32	+GlcNac	$2.3 \times 10^4 \pm 1 \times 10^3$ (62% inhibition)	nd
	GlcNac pretreatment	$2.9 \times 10^4 \pm 4 \times 10^3$ (51% inhibition)	nd
	+D-mannose	$2.7 \times 10^4 \pm 3 \times 10^3$ (55% inhibition)	nd
	D-mannose pretreatment	$3.4 \times 10^4 \pm 5 \times 10^3$ (43% inhibition)	nd
	control	$1.2 \times 10^5 \pm 2 \times 10^4$	$2.4 \times 10^3 \pm 3 \times 10^2$
	+GlcNac	$1.4 \times 10^5 \pm 1 \times 10^4$ (-)	nd
	GlcNac pretreatment	$1.5 \times 10^5 \pm 2 \times 10^4$ (-)	nd
	+D-mannose	$1.6 \times 10^5 \pm 2 \times 10^4$ (-)	nd
D-mannose pretreatment	$1.5 \times 10^5 \pm 1 \times 10^4$ (-)	nd	
<i>V. cholerae</i> O1 14034	control	$9.1 \times 10^6 \pm 0.9 \times 10^6$	$5.0 \times 10^4 \pm 7 \times 10^3$

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**Figure captions**

937

**Figure 1**

939 Temporal variation in the percentage of total (black circle), viable (black square) and  
 940 culturable (black triangle) *V. splendidus* LGP32 and *V. aestuarianus* 01/32 cells  
 941 incubated in seawater under aseptic conditions and at different temperature, salinity and  
 942 nutrient concentration: 25°C, 20‰ salinity (a); 25°C, 35‰ salinity (b); 5°C, 20‰  
 943 salinity (c), 5°C, 35‰ salinity (d); 25°C, 20‰ salinity, 40mg/L Peptone (e). Results are  
 944 the average of three replicate experiments

945

**Figure 2**

946

947 Temporal variation in the percentage of total (black circle), viable (black square) and  
948 culturable (black triangle) *V. splendidus* LGP32 and *V. aestuarianus* 01/32 cells  
949 incubated in the sediment under aseptic conditions at 5°C, 20‰ salinity. Results are the  
950 average of three replicate experiments

951

952 **Figure 3**

953 Biofilm formation of *V. splendidus* LGP32 and *V. aestuarianus* 01/32 strains on PVC  
954 surfaces at 18°C (black square) and 24°C (black triangle). Results are the average of  
955 three replicate experiments

956

957 **Figure 4**

958 Percentage of live (L) and dead (D) cells of *V. splendidus* LGP32 and *V. aestuarianus*  
959 01/32 strains calculated on total, >0.2µm and <0.2µm cell size fractions after prolonged  
960 exposure (90 days) to ASW (20‰ salinity) at 5 °C under starving condition. The  
961 number of total cells (total) at the beginning of the experiment and the number of  
962 culturable cells (culturable fraction) following incubation are also reported

963

964 **Figure 5**

965 Standard microscopic imaging (magnification 1000X) showing morphological changes  
966 observed in *V. aestuarianus* 01/32 cells following prolonged exposure to ASW (20‰  
967 salinity) at 5 °C under starving condition. Similar results were obtained for *V.*  
968 *splendidus* LGP32 strains

969

970 **Figure 6**

971 BIVALIFE experimental station in Goro lagoon (Northern Adriatic Sea, Italy)

972

973 **Figure 7**

974 Temporal occurrence of *Vibrio* spp. enumerated by culture-based (light grey bar) and  
975 molecular (dark grey bar) methods in environmental matrices and bivalves collected at  
976 the BIVALIFE experimental station in the Goro lagoon

977

978 **Figure 8**

979 Average Real-Time PCR counts of *V. splendidus* clade bacteria (a) and *V. aestuarianus*  
 980 (c) in warm and cold months in the different environmental matrices and bivalves in the  
 981 Goro lagoon during the study period (May 2011 to January 2013). Months when the  
 982 bacteria were detected are superimposed on the histogram bars

983

984 **Figure 9**

985 Presence and distribution of genes linked to environmental persistence and virulence in  
 986 environmental isolates of *V. splendidus*, *V. aestuarianus* and other *V. splendidus*-clade  
 987 bacterial species isolated from the Goro lagoon

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1000 **Supplementary Material**

1001

1002 **Supplementary Table 1**

1003 *Vibrio* strains isolated from the different environmental matrices and bivalves in the  
 1004 Goro lagoon during the study period (May 2011 to January 2013). Isolates belonging to  
 1005 the species *V. splendidus* and *V. aestuarianus* are indicated in bold. Presence (+) and  
 1006 absence (-) of genes encoding for adhesins to chitin substrates (*mshA* and *gbpA*) and  
 1007 genes encoding for virulence associated traits (*ompU* and *vsm/vam*) is also reported

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Isolate	Matrix	Date	Species	<i>mshA</i>	<i>gbpA</i>	<i>ompU</i>	<i>vsm/vam</i>
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			<i>V. splendidus</i>	+	-	+	+
			<b>LGP32</b>				
			<i>V. aestuarianus</i>	+	+	+	+
			<b>01/32</b>				
<b>165</b>	<b>Oyster</b>	<b>14/02/2012</b>	<i>V. splendidus</i>	-	-	-	-
<b>168</b>	<b>Oyster</b>	<b>21/05/2012</b>	<i>V. splendidus</i>	-	-	+	+
207A	Oyster	18/06/2012	<i>V. celticus</i>	-	-	+	-
207B	Oyster	18/06/2012	<i>V. celticus</i>	-	-	+	-
<b>162</b>	<b>Mussel</b>	<b>14/02/2012</b>	<i>V. splendidus</i>	-	-	-	-
173	Mussel	21/05/2012	<i>V. crassostreae</i>	-	+	-	+
174	Mussel	21/05/2012	<i>V. celticus</i>	-	-	+	-
202	Mussel	18/06/2012	<i>V. celticus</i>	-	-	+	+
<b>334</b>	<b>Mussel</b>	<b>14/01/2013</b>	<i>V. splendidus</i>	-	-	+	+
19	Zooplankton	30/05/2011	<i>V. celticus</i>	-	-	+	-
<b>156</b>	<b>Zooplankton</b>	<b>14/02/2012</b>	<i>V. splendidus</i>	-	-	+	-
179bis	Zooplankton	21/05/2012	<i>V. chagasii</i>	-	-	-	+
<b>180</b>	<b>Zooplankton</b>	<b>21/05/2012</b>	<i>V. splendidus</i>	-	-	+	+
<b>152</b>	<b>Sediment</b>	<b>14/02/2012</b>	<i>V. splendidus</i>	-	-	+	+
<b>153</b>	<b>Sediment</b>	<b>14/02/2012</b>	<i>V. splendidus</i>	-	-	+	-
190	Sediment	21/05/2012	<i>V. celticus</i>	-	-	+	-
<b>190bis</b>	<b>Sediment</b>	<b>21/05/2012</b>	<i>V. splendidus</i>	-	-	+	+
<b>325</b>	<b>Sediment</b>	<b>14/01/2013</b>	<i>V. splendidus</i>	-	-	+	-
2	Seawater	30/05/2011	<i>V. chagasii</i>	-	+	-	+
8	Seawater	30/05/2011	<i>V. celticus</i>	-	-	-	+
<b>147</b>	<b>Seawater</b>	<b>14/02/2012</b>	<i>V. splendidus</i>	-	-	+	+
150	Seawater	14/02/2012	<i>V. lentus</i>	-	-	+	-
151	Seawater	14/02/2012	<i>V. lentus</i>	-	-	-	-
184	Seawater	21/05/2012	<i>V. chagasii</i>	-	-	-	+
<b>185</b>	<b>Seawater</b>	<b>21/05/2012</b>	<i>V. splendidus</i>	-	-	+	+
<b>279</b>	<b>Seawater</b>	<b>03/09/2012</b>	<i>V. splendidus</i>	-	-	-	-
318	Seawater	14/01/2013	<i>V. splendidus</i>	-	-	+	-
<b>36</b>	<b>Oyster</b>	<b>30/05/2011</b>	<i>V. aestuarianus</i>	+	+	+	-
<b>38</b>	<b>Oyster</b>	<b>30/05/2011</b>	<i>V. aestuarianus</i>	-	+	+	+
<b>40</b>	<b>Oyster</b>	<b>30/05/2011</b>	<i>V. aestuarianus</i>	+	+	+	+
<b>29</b>	<b>Mussel</b>	<b>30/05/2011</b>	<i>V. aestuarianus</i>	-	+	-	+
<b>32</b>	<b>Mussel</b>	<b>30/05/2011</b>	<i>V. aestuarianus</i>	-	-	+	+
<b>33</b>	<b>Mussel</b>	<b>30/05/2011</b>	<i>V. aestuarianus</i>	-	-	+	+
<b>17</b>	<b>Zooplankton</b>	<b>30/05/2011</b>	<i>V. aestuarianus</i>	+	+	+	+
<b>18</b>	<b>Zooplankton</b>	<b>30/05/2011</b>	<i>V. aestuarianus</i>	-	+	-	+
<b>241</b>	<b>Zooplankton</b>	<b>16/07/2012</b>	<i>V. aestuarianus</i>	-	+	+	+
<b>244</b>	<b>Zooplankton</b>	<b>16/07/2012</b>	<i>V. aestuarianus</i>	-	+	+	+
<b>245</b>	<b>Zooplankton</b>	<b>16/07/2012</b>	<i>V. aestuarianus</i>	-	+	+	+
<b>287</b>	<b>Sediment</b>	<b>03/09/2012</b>	<i>V. aestuarianus</i>	-	+	+	+

1009

1010 **Supplementary Figure 1**1011 Scatter plots showing relationships existing between *V. splendidus* clade bacteria, *V.*1012 *aestuarianus*, and *Vibrio* spp. in the different environmental matrices and bivalves and

1013 sea surface temperature values in the Goro lagoon during the study period (May 2011 to  
1014 January 2013). Grey backgrounds behind plots indicate a significant correlation  
1015 ( $p < 0.05$ ) between variables.

1016

1017 **Supplementary Figure 2**

1018 Scatter plots showing relationships existing between *V. splendidus* clade bacteria, *V.*  
1019 *aestuarianus*, and *Vibrio* spp. in the different environmental matrices and bivalves and  
1020 salinity values in the Goro lagoon during the study period (May 2011 to January 2013).

1021 Grey backgrounds behind plots indicate a significant correlation ( $p < 0.05$ ) between  
1022 variables.

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1 Aquatic Ecology of the Oyster Pathogens *Vibrio splendidus* and  
2 *Vibrio aestuarianus*

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4 **\*\*REVISED\*\***

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19 Journal: Environmental Microbiology

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22 **Running title:** Ecology of *Vibrio splendidus* and *Vibrio aestuarianus*

49 **Abstract**

50 The ecology of the oyster pathogens *Vibrio splendidus* and *Vibrio aestuarianus* in the  
51 brackish aquatic environment was extensively investigated in this study. By conducting  
52 laboratory experiments under natural setting conditions it was shown that *V. splendidus*  
53 LGP32 strain generally exhibits longer persistence in both seawater and sediment than  
54 *V. aestuarianus* 01/32 strain. Both strains maintained viability and culturability for  
55 longer times in the sediment suggesting this compartment may represent a suitable  
56 niche for their persistence in the environment. In addition, both strains attached to chitin  
57 particles and copepods, the efficiency of attachment being higher in *V. splendidus* than  
58 in *V. aestuarianus*. Similarly, LGP32 strain showed a greater capability to form biofilm  
59 on PVC surfaces than 01/32 strain. LGP32 and 01/32 strains were also capable of  
60 entering a viable but nonculturable state after extended incubation at 5°C, a condition  
61 commonly found during cold season in the aquatic brackish environment. These results  
62 are consistent with field data collected during a two-year sampling campaign in the  
63 Northern Adriatic Sea, and provide background information on the mechanisms  
64 promoting *V. splendidus* and *V. aestuarianus* persistence in coastal water thus  
65 contributing to a better understanding of the epidemiology of the associated diseases.

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## 82 Introduction

83 Mollusk farming has become one of the largest aquaculture activity in the world. By  
84 volume, oysters (*Ostreidae*) are the second most important aquacultured taxonomic  
85 group to cyprinids at 4.5 million tons per year (FAO, 2013). In particular, the Pacific  
86 cupped oyster, *Crassostrea gigas*, provides the greatest contribution with 0.6 million  
87 tons per year of global production volume, valued at USD 1.3 billion in 2009 (FAO,  
88 2013).

89 Although mollusk culture is steadily growing in importance in the aquaculture sector,  
90 the European populations of cultivated mollusks are suffering from severe mortality  
91 outbreaks. From 2008, mass mortality episodes of *C. gigas* were reported in farming  
92 areas in several countries including France, Spain, the Netherlands, United Kingdom,  
93 Ireland, and the United States (Samain and McCombie, 2008). These outbreaks  
94 generally occur in summer in sheltered habitats when mortality can reach 80-100%  
95 especially in oyster juveniles. Causes triggering the outbreaks are still poorly  
96 understood and are attributed to complex interactions between oysters, pathogens and  
97 environmental parameters (Pernet et al., 2012).

98 The naturally occurring Gram-negative bacteria *Vibrio splendidus* and *Vibrio*  
99 *aestuarianus* have been associated to the summer mortalities affecting the production of  
100 *C. gigas* worldwide (Lacoste et al., 2001; Waechter et al., 2002; Le Roux et al., 2002;  
101 Gay et al., 2004a; Garnier et al., 2008). In particular, a *V. splendidus* LGP32 strain  
102 isolated from oysters suffering the “summer mortality syndrome” was shown to be  
103 highly pathogenic and cause death when injected to bivalves (Gay et al., 2004a,b; Le  
104 Roux et al., 2007). It was shown that *V. splendidus* LGP32 invades oyster immune cells,  
105 the hemocytes, through subversion of host-cell actin cytoskeleton and survives  
106 intracellularly by preventing acidic vacuole formation and limiting reactive oxygen  
107 species production (Duperthuy et al., 2010, 2011). In addition, a secreted  
108 metalloprotease (*vsm*) produced by this strain is associated with toxicity (Le Roux et al.,  
109 2007; Binesse et al., 2008). Epidemiological studies conducted during recurrent summer  
110 mortality events of *C. gigas* along the French Atlantic coast have also documented the  
111 predominance of another *Vibrio* strain, *V. aestuarianus*, that was isolated from the  
112 haemolymph of diseased animals and was related to mortality outbreaks in juvenile and  
113 adult oysters (Garnier et al., 2008; Saulnier et al., 2010). A zinc metalloprotease (*vam*)  
114 similar to that observed in *V. splendidus* is produced by this species and causes lethality

115 in *C. gigas* by impairing host cellular immune defenses (Labreuche *et al.*, 2010).  
116 Although *V. splendidus* and *V. aestuarianus* have been reported to be associated with  
117 summer mortality of Pacific cupped oyster spat and their interactions with bivalves have  
118 been investigated by a number of studies (Duperthuy *et al.*, 2010; Labreuche *et al.*,  
119 2010; Saulnier *et al.*, 2010, Duperthuy *et al.*, 2011), little is known on the ecology of  
120 these pathogens outside their bivalve host.

121 The lack of information on the aquatic ecology and lifestyle of these bacteria in coastal  
122 areas greatly limits our understanding of their role in the occurrence of mortality  
123 outbreaks and the epidemiology of such events. Vibrios are natural inhabitants of  
124 marine coastal and brackish environment throughout the world; they are equipped with  
125 a battery of adaptive response mechanisms which allow them to persist in the  
126 environment even during unfavorable conditions (*e.g.* temperature fluctuation, nutrient  
127 limitation, UV light stress, etc.) (Vezzulli *et al.*, 2009). Sea Surface Temperature (SST)  
128 is by far one of the main variables affecting the occurrence and viability of these  
129 bacteria in the aquatic environment as most vibrios show a strong seasonal oscillation  
130 with higher abundances generally observed when SST typically exceeds 18 °C (Vezzulli  
131 *et al.*, 2009). Below this temperature and especially at cold temperatures lower than 10  
132 °C, vibrios enter the viable but nonculturable (VBNC) state, a dormant condition in  
133 which cells remain viable, but are not culturable in conventional laboratory media (Xu  
134 *et al.*, 1982).

135 Several studies have clearly shown that attachment to surfaces is an integral part of the  
136 aquatic lifestyle of many vibrios, representing a successful survival mechanism  
137 (Vezzulli *et al.*, 2010). Animals with an exoskeleton of chitin, aquatic plants, protozoa,  
138 bivalves, waterbirds, as well as abiotic substrates (*e.g.* sediments) exemplify  
139 environmental matrices identified as preferential reservoirs for *Vibrio* bacteria (Pruzzo  
140 *et al.*, 2008; Vezzulli *et al.*, 2010). At molecular level, different bacterial colonization  
141 factors (*e.g.* pili and outer membrane and secreted proteins) have been described to  
142 promote *Vibrio* attachment to these substrates (Chiavelli *et al.*, 2001; Vezzulli *et al.*,  
143 2008; Stauder *et al.*, 2010). Adhesion represents the first step for substrate colonization  
144 and subsequent formation of biofilm, which, in turn, can play a major role in the  
145 persistence and transmission of these pathogens. For example, in coastal environments,  
146 *Vibrio cholerae* cells have been shown to survive year round, mostly in a non culturable  
147 state, within clusters of biofilm (Huq *et al.*, 2008). An obvious mechanism by which

148 pathogens in biofilms cause disease is by the seeding and dispersal of a large number of  
149 cells which subsequently can initiate an infection (Huq *et al.*, 2008).

150 Most of the above information on the aquatic ecology of vibrios is coming from human  
151 pathogenic species, particularly the model microorganism and etiological agent of  
152 cholera in humans, *V. cholerae*. Given the substantial lack of knowledge on life outside  
153 the host for the bivalve pathogens *V. splendidus* and *V. aestuarianus*, relevant to the  
154 FP7-European project BIVALIFE (“Controlling infectious diseases in oysters and  
155 mussels in Europe”), the objective of this study was to extensively investigate and  
156 provide solid background information on the ecology of these species, including  
157 occurrence, persistence and survival strategies, in the aquatic brackish environment.

158

## 159 **Results and Discussion**

160

### 161 **Persistence of *V. splendidus* and *V. aestuarianus* in seawater and sediments under** 162 **different conditions**

163 Seawater temperature and salinity are known to play a pivotal role in affecting the  
164 occurrence and persistence of vibrios in the aquatic environment. The optimal  
165 temperature for growth of *V. splendidus* LGP32 and *V. aestuarianus* 01/32 strains  
166 cultured under laboratory conditions ranged from 20°C to 25°C, and the optimal salinity  
167 was around 20‰. Generation time assessed by culturing the bacteria in Zobell Marine  
168 Broth at 20°C was close to 60 min for both strains.

169 Laboratory microcosm experiments were conducted to assess the persistence (evaluated  
170 in terms of maintenance of culturability and viability over time) of *V. splendidus* LGP32  
171 and *V. aestuarianus* 01/32 strains in both seawater and sediments under experimental  
172 conditions mimicking those found in coastal waters (5°C and 25°C temperature, 20‰  
173 and 35‰ salinity, as usually recorded in such environments over the seasonal cycle).  
174 Following these settings, it was observed that *V. splendidus* LGP32 strain generally  
175 exhibited higher persistence in both seawater and sediments than *V. aestuarianus* 01/32.  
176 In seawater, the majority of *V. splendidus* bacteria maintained viability over a period of  
177 25 days at 5°C, whilst at 25°C a decrease in viability was observed after 10 days  
178 incubation (**Fig. 1a,b**). The different response at the two temperatures may likely be  
179 related to an imbalance between the metabolic rate (higher at 25°C than at 5°C) and  
180 nutrient availability. *V. splendidus* bacteria generally lost culturability in seawater after

181 a short incubation time (less than 5 days) in all tested conditions. After this time, the  
182 bacteria appeared bright green, very small and coccoid in shape when viewed by  
183 fluorescence-based Live/Dead assay with epifluorescence microscopy thus likely  
184 entering a VBNC physiological state. Unlike *V. splendidus* LGP32, *V. aestuarianus*  
185 01/32 strain lost both viability and culturability in seawater within 5 days incubation in  
186 almost all experimental settings investigated in this study (**Fig. 1**). This suggests that *V.*  
187 *aestuarianus* 01/32 is possibly more demanding in terms of living conditions than *V.*  
188 *splendidus* LGP32. Such conditions are probably not met by simplified laboratory  
189 settings whilst they might be possibly satisfied in the more complex natural  
190 environment (*e.g.* by the presence of nutrients). According to this, initial addition of  
191 nutrients (40 mg/L Peptone) to experimental microcosms kept at 25°C and 20‰ salinity  
192 prolonged the maintenance of viability and cultivability of both strains (**Fig. 1e**).

193 In the sediment, results on persistence and survival of the two *Vibrio* species resembled  
194 those observed in seawater. However, in this compartment, the level of viability (*V.*  
195 *aestuarianus*) and culturability (*V. splendidus* and *V. aestuarianus*) was generally  
196 higher throughout the entire duration of the experiment and never dropped below 10<sup>4</sup>  
197 cells/ml. At least in static conditions, the sediment thus represents a more suitable  
198 matrix for the persistence of *V. splendidus* LGP32 and *V. aestuarianus* 01/32 in the  
199 aquatic environment (**Fig. 2**). Accordingly Johnson *et al.*, 2010 observed a protective  
200 effect in sediment, compared to oyster and water, for *V. parahaemolyticus* and *V.*  
201 *vulnificus*. This is also probably linked to the fact that sediment provides biotic and  
202 abiotic surfaces useful for bacterial biofilm development; moreover concentration of  
203 organic matter in this compartment is higher than in the overlying water column (10.000  
204 to 100.000-fold higher in natural conditions, Vezzulli *et al.*, 2009).

205

### 206 **Survival strategies in the aquatic environment: adhesion to environmental chitin** 207 **surfaces**

208 The mechanisms promoting *V. splendidus* and *V. aestuarianus* persistence in the  
209 aquatic environment is an issue deserving investigation since it may be crucial for  
210 understanding the occurrence, distribution and survival of these bacteria and the  
211 epidemiology of the bivalve morbidity and mortality. To this aim, the capability of *V.*  
212 *splendidus* LGP32 and *V. aestuarianus* 01/32 strains to interact with both chitin  
213 particles and plankton crustaceans (*Tigriopus fulvus* copepods) was analyzed *in vitro*.



214 Since *V. cholerae* adhesion to chitin substrates has been extensively investigated and  
215 the main related mechanisms have been well characterized (Pruzzo *et al.*, 2008), *V.*  
216 *cholerae* O1 El Tor 14034 attachment to both substrates was also assessed as a  
217 reference and used for comparison. Adhesion assays showed that *V. splendidus* and *V.*  
218 *aestuarianus* strains attached to both tested substrates. *V. splendidus* LGP32 showed a  
219 higher efficiency in attachment to chitin particles and copepods than *V. aestuarianus*  
220 01/32 strain (T-test,  $p < 0.05$ ) (**Table 1**). However, both strains were less adhesive than  
221 *V. cholerae* 14034 toward the tested substrates (T-test,  $p < 0.05$ ) (**Table 1**).

222 In order to preliminary assess the nature of the ligands mediating LGP32 and 01/32  
223 attachment to environmental chitin surfaces, the presence of genes encoding for known  
224 chitin binding adhesins was studied. In particular, we analyzed the presence of two  
225 ligands mediating the attachment of *V. cholerae* to environmental chitin surfaces: the  
226 mannose-sensitive haemagglutinin (MSHA) pilus and the N-acetylglucosamine  
227 (GlcNAc)-binding protein A (GbpA). The former is a type 4 pilus mediating binding to  
228 D-mannose-containing receptors and facilitating adhesion to chitin independent of the  
229 surface chemistry (Meibom *et al.*, 2004), whereas the latter is a cell surface protein  
230 which specifically recognizes GlcNAc residues (Stauder *et al.*, 2012). We found the  
231 *mshA* gene in both strains and the *gbpA* gene only in *V. aestuarianus* 01/32 strain.  
232 Experiments of adhesion to chitin and copepods were then performed in the presence of  
233 GlcNAc and D-mannose that in *V. cholerae* act as inhibitors of interactions with chitin  
234 containing substrates mediated by GbpA and MSHA, respectively. As shown in **Table**  
235 **1**, in the presence of these sugars, the level of *V. aestuarianus* 01/32 attachment to both  
236 chitin particles and copepods was significantly lower than in the controls (T-test,  
237  $p < 0.05$ ) with percentage of inhibition ranging from 43% to 62%, thus supporting the  
238 role of both ligands in attachment of this strain to chitin containing substrates. In  
239 contrast, neither the addition nor pretreatment of *V. splendidus* LGP32 cells with the  
240 sugars reduced the level of bacterial attachment to chitin and copepods. These results  
241 point out to a non significant contribution of MSHA and GlcNAc binding ligands to  
242 LGP32 interactions with chitin substrates. We can speculate that adhesins binding to  
243 GlcNAc oligomers and/or non specific mechanisms, such as those mediated by  
244 hydrophobic forces and electrostatic bonds, are involved in the process. Recently, the  
245 major outer membrane protein OmpU of *V. splendidus* LGP32 was shown to play a role  
246 in the attachment and invasion of oyster hemocytes (Duperthuy *et al.*, 2011). However,

247 to date, the involvement of this protein in the attachment to environmental substrates  
248 (*e.g.* chitin surfaces) is still unknown.

249

### 250 **Survival strategies in the aquatic environment: biofilm formation**

251 Adhesion of *Vibrio* cells to solid substrates such as environmental chitin surfaces can  
252 lead to the formation of biofilm. No information is currently available on the capability  
253 of *V. splendidus* and *V. aestuarianus* to form biofilms; however, studies conducted on  
254 other *Vibrio* species such as *V. cholerae* have clearly shown that biofilm constitutes a  
255 successful survival mechanism increasing the ability of the bacteria to persist under  
256 diverse environmental conditions. (Hall-Stoodley and Stoodley, 2005; Matz *et al.*,  
257 2005). We investigated biofilm formation of *V. splendidus* LGP32 and *V. aestuarianus*  
258 01/32 strains on PVC surfaces at temperatures (18°C and 24°C) relevant in estuarine  
259 and marine environments (**Fig. 3**). As for adhesion assays, *V. cholerae* O1 El Tor 14034  
260 was also tested. For the purpose of comparative analysis, arbitrarily defined categories  
261 were used (see “Experimental procedures”). Such categories classified bacteria as non-  
262 producer, weak producer, and strong producer.

263 At both temperatures, *V. splendidus* LGP32 strain showed a greater capability to form  
264 biofilm on PVC surfaces over the course of the experiment (strong producer with  
265 average  $OD_{545}=0.40\pm 0.02$  at 18°C and average  $OD_{545}=0.36\pm 0.03$  at 24°C) than *V.*  
266 *aestuarianus* 01/32 strain (non-producer with average  $OD_{545}=0.05\pm 0.04$  at 18°C and  
267 average  $OD_{545}=0.02\pm 0.01$  at 24°C). Both strains showed lower capability to form  
268 biofilm than *V. cholerae* 14034 ( $OD_{545}=0.8\pm 0.08$  at 18°C;  $OD_{545}=0.8\pm 0.08$  at 24°C).  
269 These results might help to explain higher persistence of *V. splendidus* LGP32 than *V.*  
270 *aestuarianus* 01/32 strain observed in the sediment by laboratory experiments.

271

### 272 **Survival strategies in the aquatic environment: entering into the viable but 273 nonculturable (VBNC) state**

274 Since VBNC state in vibrios is commonly induced by temperatures below 10°C (Oliver,  
275 2005), we studied the effect of prolonged exposure (90 days) of *V. splendidus* LGP32  
276 and *V. aestuarianus* 01/32 strains to artificial sea water (ASW 20‰ salinity) at 5 °C  
277 under starving condition.

278 Incubation of bacteria under such conditions resulted in different cell responses by the  
279 two *Vibrio* strains. After 90 days incubation, the number of CFU/ml drastically dropped

280 to  $10^1$  CFU/ml, corresponding to 0,00001% of total cells, for *V. aestuarianus* 01/32  
281 strain, and  $10^4$  CFU/ml, corresponding to 0,01% of total cells, for *V. splendidus* LGP32  
282 (**Fig. 4**). Consistent with results from laboratory microcosm experiments (**Fig. 1**),  
283 46,8% of *V. splendidus* LGP32 cells retained viability after incubation (as determined  
284 by fluorescence-based Live/Dead assay for cell viability), whilst the majority of *V.*  
285 *aestuarianus* 01/32 cells (94%) appeared dead after prolonged exposure to low  
286 temperature in ASW (20‰ salinity) (**Fig. 4**).

287 In order to detect the presence of VBNC cells, the presence and relative abundance  
288 (expressed as percentage of total cells) of viable and culturable bacteria was also  
289 investigated on different cell size fractions ( $> 0.2\mu\text{m}$  and  $< 0,2\mu\text{m}$ ) obtained by filtering  
290 the bacterial suspensions through polycarbonate filters of  $0,2\ \mu\text{m}$  pore size. Only  
291 fractions of *V. splendidus* and *V. aestuarianus* containing bacterial cells larger than  $0.2$   
292  $\mu\text{m}$  yielded colonies on culture media (**Fig. 4**). In contrast, for both strains, cells smaller  
293 than  $0.2\ \mu\text{m}$  completely lost culturability ( $<0.1$  CFU/ml) (**Fig. 4**). Retention of  
294 membrane integrity of this cell fraction was investigated using the fluorescence-based  
295 Live/Dead assay for cell viability. It was shown that the majority of the cells ( $>70\%$ )  
296 indeed retained viability. Contamination was ruled out by testing DNA extracted from  
297 *V. splendidus* and *V. aestuarianus* bacteria smaller than  $0.2\ \mu\text{m}$  with specific primers.  
298 Interestingly, VBNC bacteria maintained the same virulence related genes found in the  
299 parents (*mshA*, *ompU* and metalloprotease genes for *V. splendidus* LGP32 and *mshA*,  
300 *gbpA*, *ompU* and metalloprotease genes for *V. aestuarianus* 01/32). In addition,  
301 observation by both standard and epifluorescence microscopy showed that bacteria  
302 besides becoming smaller in size changed from rod to ovoid or coccoid morphology  
303 (**Fig. 5**).

304 Overall, these results provide evidence that *V. splendidus* LGP32 and, to a lesser extent,  
305 *V. aestuarianus* 01/32 are capable of entering a VBNC physiological state after  
306 extended incubation at  $5^\circ\text{C}$ , a condition that could be met during the winter season in  
307 shallow brackish environment where bivalve farming generally takes place. Besides  
308 contributing to the bacterial persistence in such environments, bacterial cells in the  
309 VBNC state might also retain virulence properties and might re-activate under favorable  
310 conditions into an actively metabolizing and culturable form, as shown in earlier studies  
311 for other *Vibrio* species (Pruzzo *et al.*, 2003). Studies in this direction are currently  
312 undergoing in our laboratory.

313

314 **Occurrence, temporal variations and environmental reservoirs of *V. splendidus* and**  
315 ***V. aestuarianus* in the Goro Lagoon (Northern Adriatic Sea, Italy)**

316 In order to complement and validate the results obtained by laboratory experiments, the  
317 occurrence and temporal variations of *V. aestuarianus* and *V. splendidus*-clade bacteria  
318 were investigated in the Goro Lagoon (Northern Adriatic Sea, Italy) (**Fig. 6**) in different  
319 environmental matrices (seawater, sediment, zooplankton) from May 2011 to January  
320 2013. Identification and enumeration of these bacteria were conducted in parallel in  
321 both mussels (*Mytilus galloprovincialis*) and oysters (*C. gigas*) collected at the same  
322 location. In addition, the concentration of *Vibrio* spp. bacteria was also assessed in order  
323 to evaluate the general ecological features of the *Vibrio* community in the study area.

324 As shown in **Fig. 7**, the number of total and culturable *Vibrio* spp. bacteria did not show  
325 a significant temporal trends in environmental matrices and bivalves. Interestingly, high  
326 number of culturable *Vibrio* spp. were found also in winter months (February 2012 and  
327 January 2013 SST<5°C) in contrast to what it is commonly observed in temperate  
328 coastal environment where vibrios can be generally cultured only during the warm  
329 season (Johnson *et al.*, 2010; Oliver *et al.*, 2013). No significant correlation was found  
330 between SST and *Vibrio* spp. concentration in all environmental matrices with the  
331 exception of zooplankton where a significant positive correlation with temperature was  
332 observed (Pearson correlation analysis,  $p < 0.05$ ) (**Supplementary Fig. 1**). The absence  
333 of a clear temporal trend in *Vibrio* spp. abundance as well as the presence of culturable  
334 vibrios in seawater during cold months in the Goro Lagoon could be related to the high  
335 nutrients content of these waters (Viaroli *et al.*, 2006). This condition is known to favor  
336 bacterial persistence and maintenance of culturability in *Vibrio* species (Oliver, 2005).  
337 Nevertheless, the number of *Vibrio* spp. determined by Real-Time PCR was at least one  
338 order of magnitude greater than culturable counts also suggesting the presence of a large  
339 fraction of dead and/or VBNC bacterial cells in the studied environment (**Fig. 7**).

340 *V. aestuarianus* and *V. splendidus*-clade bacteria were found in the lagoon by Real Time  
341 PCR studies in association with a number of environmental matrices and living  
342 organisms that may function as reservoirs and/or hosts. In particular, higher  
343 concentrations of *V. splendidus*-clade bacteria were associated to zooplankton compared  
344 to the other environmental matrices, with values generally exceeding  $10^6$  cells/g both in  
345 warm and cold months (**Fig. 8**). These bacteria were also frequently detected in oysters

346 and mussels; when present, their concentration ranged from  $1.1 \times 10^5$  to  $1.5 \times 10^7$  cells/g  
347 and from  $2.8 \times 10^2$  to  $3.8 \times 10^7$  cells/g for oyster and mussel respectively.

348 In seawater and sediment compartments, when present, their concentration ranged from  
349  $1.9 \times 10^1$  to  $7.6 \times 10^2$  cells/ml and from  $7.4 \times 10^2$  to  $1.6 \times 10^5$  cells/g, respectively. The  
350 sediment was the compartment where *V. splendidus*-clade bacteria were more  
351 frequently found during the study period (9 out of 11 sampling dates) and may thus  
352 represent a suitable niche for their persistence in the environment. *V. splendidus*-clade  
353 bacteria were also found associated to a variety of other environmental substrates  
354 commonly found in the lagoon including large benthic crustaceans (*Dyspanopeus sayi*,  
355 *Upogebia pusilla*, *Cratigon* sp., *Carcinus aestuarii*), *Gracilaria verrucosa* macroalgae  
356 and *Tapes philippinarum* clam (data not shown).

357 In contrast to *V. splendidus*, *V. aestuarianus* cells were rarely detected in the Goro  
358 lagoon samples. In particular, high concentrations of *V. aestuarianus* bacteria were  
359 found associated to zooplankton during warm months, with values ranging from  $1.4 \times 10^5$   
360 to  $6.9 \times 10^6$  cells/g, and only sporadically found in mussels, oysters, seawater and  
361 sediments (**Fig. 8**). In particular, during cold months, *V. aestuarianus* was detected only  
362 once (January 2013) in the sediment compartment. The sediment may thus represent an  
363 environmental reservoir also for this species where, in accordance with results from  
364 laboratory experiments (**Fig. 2**), the bacteria can find a favorable environment for  
365 overwintering (Vezzulli *et al.*, 2009).

366 Interestingly data on the survival of *V. splendidus* LGP32 and *V. aestuarianus* 01/32 in  
367 *C. gigas* and *M. galloprovincialis* showed that both strains are resistant to killing by  
368 oyster hemolymph (serum + hemocytes) while *V. aestuarianus* only is sensitive to  
369 killing by *M. galloprovincialis* hemocytes (Balbi *et al.*, 2013).

370 Culture based analyses showed that *V. splendidus*-clade bacteria are often present in  
371 culturable form in the different matrices. Sequencing of *pyrH* gene of these isolates  
372 showed that half of them belonged to the *V. splendidus* species (2 from oysters, 2 from  
373 mussels, 2 from zooplankton, 4 from sediment and 4 from seawater) suggesting that  
374 bacteria belonging to *V. splendidus* species, as well as those belonging to *V. splendidus*  
375 clade, are widespread in the Goro lagoon and can maintain culturability also during cold  
376 months (**Supplementary Table 1**). Regarding culturable *V. aestuarianus* bacteria, they  
377 were found only sporadically in warm months. A total of 12 strains was isolated, 3 from  
378 oysters, 3 from mussels, 5 from zooplankton and 1 from sediment. These results,

379 besides being consistent with those obtained by Real-Time PCR, also support what  
380 observed in laboratory microcosms, *i.e.* higher capability to persist in the environment  
381 showed by *V. splendidus* in comparison with *V. aestuarianus*, which rapidly loses  
382 culturability in all tested conditions.

383 Overall, environmental variables such as SST and salinity do not seem to have a  
384 significant influence on the presence and concentration of these bacteria in  
385 environmental matrices and bivalves (**Supplementary Fig. 1, 2**). In agreement to what  
386 we observed for the whole *Vibrio* spp. community, this may be related to the ecological  
387 features of the Goro lagoon; in fact it is well known that in shallow coastal eutrophic  
388 environment microbial abundance might lack a clear seasonal trend being marginally  
389 subjected to the influence of the main environmental variables (Vezzulli and Fabiano,  
390 2006; Moreno *et al.*, 2008, 2011). Indeed, a significant positive correlation was only  
391 found between SST and *V. splendidus*-clade/*V. aestuarianus* bacteria associated to  
392 zooplankton (Pearson correlation analysis,  $p < 0.05$ ), a condition which has already been  
393 described in other *Vibrio* species such as *V. cholerae* (Stauder *et al.*, 2010)  
394 (**Supplementary Fig. 1**). Surprisingly, a significant negative correlation was found  
395 between SST and *V. splendidus*-clade bacteria in seawater (Pearson correlation analysis,  
396  $p < 0.05$ ). A full explanation for this is not available, however this result is consistent to  
397 observations made in other oyster farming sites across Europe (Chris Roger, personal  
398 communication).

399

#### 400 **Occurrence of genes linked to environmental persistence and virulence in *V.*** 401 ***splendidus* and *V. aestuarianus* isolates from the Goro lagoon**

402 To evaluate the presence and distribution of genes linked to environmental persistence  
403 and virulence in environmental isolates from the Goro lagoon, genes encoding for  
404 MSHA, GbpA, OmpU and zinc metalloprotease were evaluated in 39 strains including  
405 12 *V. aestuarianus* and 14 *V. splendidus* isolates. The remaining isolates belonged to  
406 other species of the *V. splendidus* clade (**Supplementary Table 1**).

407 Regarding genes encoding for adhesins involved in attachment to chitin, all *V.*  
408 *splendidus* isolates lacked both *mshA* and *gbpA*. In contrast, almost all *V. aestuarianus*  
409 strains (10 out of 12) were positive for *gbpA* and three strains carried the *mshA* gene  
410 (**Fig. 9**). These results suggest that adhesion ligands, mainly GbpA, are diffused among  
411 lagoon populations of *V. aestuarianus* and their expression might contribute to the

412 persistence of these bacteria in the environment through chitin surface colonization. The  
413 fact that they are not found among *V. splendidus* species supports the possible role of  
414 other unknown ligands in colonization of environmental substrates.

415 As regards to genes associated to virulence (Le Roux *et al.*, 2007; Labreuche *et al.*,  
416 2010; Duperthuy *et al.*, 2011), most *V. splendidus* strains were observed to carry the  
417 *ompU* gene (11 out of 14) whilst the zinc metalloprotease gene was detected in half of  
418 the isolates (7 out of 14). In the case of *V. aestuarianus*, the majority of isolates was  
419 positive for OmpU (10 out of 12) and zinc metalloprotease (11 out of 12) genes (**Fig. 9,**  
420 **Supplementary Table 1**). Interestingly, genes encoding OmpU protein and zinc  
421 metalloprotease were also detected in isolates belonging to other species closely related  
422 to *V. splendidus* including *V. celticus*, *V. crassostrea*, *V. chagasii* and *V. lentus* (**Fig. 9,**  
423 **Supplementary Table 1**).

424 These data suggest that virulence-related genes are widely diffused in the environment  
425 not only in the species potentially pathogenic for oysters but also in other harmless  
426 bacterial species. Such strains might thus represent a reservoir of these genes in the  
427 aquatic environment that might be exchanged among *Vibrio* strains by horizontal gene  
428 transfer (HGT) (Sechi *et al.*, 2000).

429

### 430 **Concluding remarks**

431 Reducing the impact of environmental pathogens mostly relies on knowledge of their  
432 biology and ecology including the mechanisms that allow their survival outside the host.  
433 Marine bivalves such as oysters are typically reared in shallow brackish waters  
434 frequently subjected to fluctuations of environmental factors such as temperature,  
435 salinity and nutrient concentration. Using both *in vitro* and in field studies, we provided  
436 evidence that the oyster pathogens *V. splendidus* and *V. aestuarianus* persist in the  
437 aquatic environment with different efficiency by adopting various survival strategies,  
438 including adhesion to environmental chitin substrates and biofilm formation.  
439 Zooplankton and the sediment compartment seem to represent important environmental  
440 reservoirs where these bacteria can persist in high abundance and/or can be found  
441 during cold and unfavorable environmental conditions. Both *V. splendidus* and *V.*  
442 *aestuarianus* are also capable of entering a VBNC state after extended incubation at  
443 5°C, a condition commonly found during cold season in the aquatic brackish  
444 environment. Interestingly, it was observed that a significant fraction of VBNC cells

445 showed a reduced size ( $<0.2 \mu\text{m}$ ) which may have important implication for their  
446 detection in the environment (*e.g.*, the use of standard membranes might miss this  
447 fraction and this should be taken into account in microbiological analysis). Overall,  
448 these data provide new background information on the mechanisms promoting *V.*  
449 *splendidus* and *V. aestuarianus* persistence in coastal water thus contributing to a better  
450 understanding of the epidemiology of their associated diseases.

451

## 452 **Experimental procedures**

453

### 454 ***In vitro* studies**

#### 455 ***Bacterial strains and culture conditions***

456 *V. splendidus* LGP32 and *V. aestuarianus* 01/32 strains kindly provided by Tristan  
457 Renault (IFREMER, La Tremblade, France) were used in this study for laboratory  
458 experiments. Both strains were cultivated aerobically for 24-48 h at 20°C° in  
459 Zobell Marine Broth 2216 (Difco Laboratories Inc.). *V. splendidus* LGP32 was also  
460 cultured in Luria–Bertani (LB) agar (Conda laboratories, Madrid, Spain) containing  
461 3% NaCl and *V. aestuarianus* 01/32 in Marine agar 2216 (Difco Laboratories Inc.). *V.*  
462 *cholerae* O1 biovar El Tor strain ATCC 14034 was also used in some experiments and  
463 cultured in LB agar and broth, or Zobell Marine Broth 2216. ASW was obtained by  
464 reconstituting Sea Salts (Sigma–Aldrich, Milan, Italy) with demineralized hypo-osmolar  
465 water to 20‰ and 35‰ final concentration and then autoclaving the resulting solution.  
466 If not differently specified, with the acronym ASW we refer to 35‰ salinity condition  
467 throughout the manuscript.

468

#### 469 ***Setting of experimental microcosms for “survival” and “VBNC induction”***

##### 470 ***experiments***

471 Microcosm vessels for survival experiments of *V. splendidus* LGP32 and *V.*  
472 *aestuarianus* 01/32 strains in seawater were prepared using sterile flasks added with 500  
473 ml of ASW and kept under aseptic conditions at different temperatures and salinity  
474 (25°C, 20‰ salinity; 25°C, 35‰ salinity; 5°C, 20‰ salinity; 5°C, 35‰ salinity). Some  
475 experiments were also performed in the presence of 40 mg/L Peptone (Sigma–Aldrich,  
476 Milan, Italy). For survival experiments in the sediment, additional flasks were added  
477 with 10 g of autoclaved sterile sediment collected in the Goro lagoon (see below) and



478 kept at 5°C under aseptic conditions. The sediment layer was gently covered with ASW  
479 and vessels were let to settle for 24 h before starting the experiments.

480 Single colonies of bacterial strains were picked from agar plates, transferred to 100 ml  
481 of Zobell Marine Broth 2216, and incubated at 20°C. Bacterial cells were harvested by  
482 centrifugation at 4500 x g for 10 min. After centrifugation, the cells were washed twice  
483 with ASW and suspended in the microcosm flasks, prepared as described above at a  
484 final concentration of 10<sup>7</sup> cells/ml(g), as determined by total epifluorescence counts  
485 (Hobbie *et al.*, 1977). Three replicate flasks were prepared for each strain and condition.  
486 Flasks were then regularly monitored for culturability and viability at day 0, 5, 10, 15  
487 and 20 (see methods described on the below).

488 For VBNC induction experiments, additional ASW (20‰ salinity) microcosm flasks  
489 (three replicate flasks for each strain) were inoculated as described above and were  
490 maintained at 5°C, a condition know to induce the VBNC state in other *Vibrio* species  
491 (Oliver *et al.*, 2010). After 3 months, the total, culturable and viable cells present in  
492 each microcosm were determined (see methods described on the below). Bacterial cells  
493 were then divided into two size fractions (>0.2 µm and <0.2µm) by passages through a  
494 0.2 µm nucleopore filter (Millipore, Milan, Italy). Total, culturable and viable cells of  
495 each size fraction were also evaluated.

496

#### 497 ***Nucleic acid extraction***

498 DNA from bacterial cultures and microcosm water samples was extracted with the High  
499 Pure Polymerase Chain Reaction (PCR) Template Preparation Kit (Roche Diagnostics,  
500 Mannheim, Germany) according to the manufacturer's instructions. Nucleic acids from  
501 sediment samples were extracted with the UltraClean soil DNA kit (MoBio  
502 Laboratories, Solana Beach, CA, USA) according to the manufacturer's instructions and  
503 recommendation provided for the use with marine sediment (Luna *et al.*, 2006). The  
504 amount of extracted DNA was quantified using the Quantifluor double-stranded DNA  
505 quantification kit (Promega Italia, Milan, Italy).

506

#### 507 ***Propidium monoazide (PMA) treatment of bacterial cells in microcosm samples***

508 Propidium monoazide (PMA) (Gentaur molecular product, Milan, Italy) was dissolved  
509 in 20% dimethyl sulfoxide to create a stock concentration of 20 mM and stored at -20°C  
510 in the dark. A first set of trial experiments was conducted for setting optimal protocols

511 to be employed in the study using variable concentrations of PMA and light exposure  
512 times.

513 According to the results, 500 µl of seawater and sediment suspensions (1 g of sediment  
514 were resuspended in 5 ml ASW, vigorously vortexed for 30 s and treated in a sonication  
515 bath for 60 s for a total of three times) were treated with PMA to final concentrations of  
516 50 µM and 100 µM for water and sediment, respectively. Following 5 min incubation in  
517 the dark with occasional mixing, samples were light exposed for 2 min using a 650W  
518 (3200K) halogen light source (Uniquartz, FIEF Lighting, Ferrara, Italy). The sample  
519 tubes were placed about 20 cm from the light source and were laid horizontally with  
520 gentle shaking on ice to avoid excessive heating. After photo-induced cross-linking,  
521 cells were pelleted at 4.500 x g for 10 min prior to DNA isolation as previously  
522 described.

523

#### 524 ***Enumeration of total and viable bacterial cells by Real-Time PCR***

525 Real-time PCR for the enumeration of *V. splendidus* LGP32 and *V. aestuarianus* 01/32  
526 in seawater and sediment samples from experimental microcosms were performed using  
527 a Taqman Real-Time PCR protocol with the LightCycler (Roche Diagnostics, Mannheim,  
528 Germany). *V. splendidus* clade (16SspF2 ATCATGGCTCAGATTGAACG; 16SspR2  
529 CAATGGTTATCCCCACATC; 16S probe FAM-  
530 CCCATTAACGCACCCGAAGGATTG-BHQ1; IFREMER, 2013) and *V. aestuarianus*  
531 (DNAj F GTATGAAATTTAACTGACCCACAA; DNAjR  
532 CAATTTCTTTTGAACAACCAC; DNAj probe FAM-  
533 TGGTAGCGCAGACTTCGGCGAC – BHQ2; IFREMER, 2013) specific primers and  
534 probe were used in the assays. Briefly, each reaction mixture contained 1X LighCycler  
535 Taqman master (Roche Diagnostics, Mannheim, Germany) and 1 µM of each primer  
536 and 0.1 µM of each probe in a final volume of 20 µl. The PCR program used was as  
537 follows: initial denaturation at 95°C for 10 min, subsequent 45 cycles of denaturation at  
538 95°C for 10 s, annealing at 60°C for 15 s and elongation at 72°C for 1 s, followed by  
539 final elongation at 72°C for 10 min. Five µl of DNA template (DNA concentration for  
540 all samples varied from 1 to 10 µg/ml) were added to the reaction mixture. Accurately  
541 quantified copy number genomic DNA of *V. splendidus* LGP32 and *V. aestuarianus*  
542 01/32 strains was used as a standard. For quantification, the log of the number of  
543 genome units (GI) of a dilution series of the sample was plotted *versus* the cycle number

544 at which the fluorescent signal increased above background or threshold (Ct value).  
545 This approach was used because, for detection and enumeration of bacterial cells in  
546 environmental samples, it is easier to understand results in actual cell numbers than in  
547 DNA concentrations or gene copy numbers. If necessary, a correction factor was  
548 applied to account for over-/under-estimation due to the effect of different efficiencies  
549 between the standard and sediment samples.

550

#### 551 ***Enumeration of total and viable bacterial cells by Epifluorescence microscopy***

552 Total (TBN) and viable (VBN) bacterial cells were enumerated in seawater samples  
553 from VBNC induction experiments using epifluorescence microscopy. TBN were  
554 assessed using the Acridine Orange Direct Count technique of Hobbie *et al.* (1977)  
555 whilst VBN were evaluated using the Live/Dead BacLight Bacterial Viability kit for  
556 microscopy and quantitative assays (Molecular Probes, Eugene, OR) following the  
557 manufacturer's instructions. Fluorescence from the stained cells was viewed using an  
558 epifluorescence microscope equipped with filter set 9 (Excitation 450-490; Beam  
559 Splitter FT510, Emission LP520) and filter set 43 (Excitation 545-25; Beam Splitter  
560 FT570, Emission BP605/70) (Zeiss Universal Microscope).

561

#### 562 ***Enumeration of culturable bacterial cells***

563 To enumerate culturable bacteria in samples from experimental microcosms, 200 µl of  
564 seawater and sediment suspension (1 g of sediment was resuspended in 5 ml ASW,  
565 vigorously vortexed for 30 s and treated in a sonication bath for 60 s for a total of three  
566 times) were serial diluted ( $10^{-1}$  to  $10^{-8}$ ) with ASW and spread in triplicate onto  
567 appropriate culture media. Plates were incubated at 20°C for 48 h and bacterial colonies  
568 were counted. Results were expressed as CFU/ml(g) (colony-forming units per  
569 milliliter).

570

#### 571 ***Adhesion assays***

572 To assess bacterial attachment to chitin particles one volume bacterial suspension  
573 ( $5 \times 10^7$  cell/ml) was added to UV-sterilized chitin purified from crab shell (2.5 mg;  
574 Sigma Chemical Co., St Louis, MO, USA) and the mixture was incubated for 1 h at  
575 20°C with shaking. A sample without chitin was also prepared as a control. After  
576 incubation, chitin particles were repeatedly washed (x3) with 1 ml ASW to remove

577 unattached bacteria. 200  $\mu$ l of PCR grade water was added to chitin pellets and DNA  
578 was extracted by boiling. The number of attached bacteria was determined by Real  
579 Time PCR using protocols described on the above. In addition, to preliminary define the  
580 nature of bacterial ligands involved in interactions with chitin, adhesion assays were  
581 also performed by either pretreating bacteria with or in the presence of sugars (GlcNAc  
582 and D-mannose) at the final concentration of 10 mg/ml (1h incubation at room  
583 temperature with shaking).

584 To assess bacterial attachment to copepods a similar protocol was followed. *T. fulvus*  
585 copepods belonging to the *Harpacticoida* family were cultured in filter-sterilized ASW  
586 and fed with unicellular phytoplanktonic algae (*Tetraselmis suecica*). Before the  
587 association assay, the copepods were vigorously washed 10 times with ASW to remove  
588 most adhering bacteria and heat killed (65°C per 15 min). One ml of bacterial  
589 suspension ( $1 \times 10^8$  cell/ml) was then added to 10 washed copepods in a tube and  
590 incubated at room temperature over night. After overnight incubation, copepods were  
591 collected and gently washed three times with phosphate buffered saline (PBS) solution  
592 (137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) PBS to remove non-adherent  
593 bacteria. 200  $\mu$ l of PBS was then added to the washed copepods and DNA was extracted  
594 as previously described. The number of attached bacteria were determined by Real  
595 Time PCR. All adhesion assays were performed in triplicate.

596

### 597 ***Biofilm assays***

598 For biofilm formation analysis, the microtiter plate method (Stepanovic *et al.*, 2000)  
599 was followed with few modifications. Overnight bacterial cultures grown in  
600 Zobell Marine Broth 2216 were diluted to  $Ab_{540} = 0.02$ . Sterilized microtitre plates (96  
601 wells) made of PVC were previously conditioned with 200  $\mu$ l ASW (overnight  
602 incubation at room temperature). Each well was filled with 180  $\mu$ l Zobell medium and  
603 20  $\mu$ l of the cell suspension and incubated for 24, 48, 72 h and 168 h at 18°C and 24°C.  
604 Biofilm was gently washed to eliminate non-adherent bacteria and stained with 0.01%  
605 crystal violet (100  $\mu$ L). After 15 min incubation at room temperature, wells were  
606 gently washed with water, and bound dye was extracted from stained cells by adding  
607 200  $\mu$ l of ethanol : acetone (8:2) per well. After about 1 h incubation, biofilm formation  
608 was quantified by measuring the absorbance (Abs) of the solution at 545 nm.

609 For the purposes of comparative analysis, we classified bacteria into three arbitrarily  
610 defined categories based upon  $Abs_{545}$  of the supernatant as follow:  $Abs_{545} \leq 0.150 =$   
611 non-producer;  $Abs_{545} \geq 0.250 =$  strong producer;  $0.150 < Abs_{545} < 0.250 =$  weak  
612 producer. Tests were done in triplicate on three separate occasions and the results  
613 averaged.

614

## 615 **In field studies**

### 616 *Experimental design and field sampling*

617 Studies in the field were carried out in the Sacca di Goro lagoon (Northern Adriatic Sea,  
618 Italy). The lagoon at the south of the Po River Delta is among the most important  
619 shellfish aquaculture systems in Italy and it has been extensively studied by previous  
620 investigators (Viaroli *et al.*, 2006). The total surface area is 830 km<sup>2</sup> for the watershed  
621 and 26 km<sup>2</sup> for the lagoon, respectively. The watershed is exploited for agriculture,  
622 whilst the coastal lagoon is one of the most important European sites for bivalve  
623 farming. The lagoon is a sheltered and shallow area (less than 5 m average depth) and it  
624 is characterized by a low circulation regime and temperatures ranging from 4°C in  
625 winter to 30°C in summer.

626 An experimental station within the FP7-European project BIVALIFE was established in  
627 the inner part of the lagoon for the cultivation of mussel *M. galloprovincialis* and oyster  
628 *C. gigas* (**Fig. 6**).

629 In 2011 commercial French seed of *C. gigas* triploids about 6 month old and indigenous  
630 diploid *M. galloprovincialis* of similar size (>600 individuals per species) were placed  
631 in alternate sectors of Ostrega<sup>tm</sup> baskets (20 for each species) which were subsequently  
632 piled and suspended in shallow water (0.5-1 m depth) of the Goro lagoon. The baskets  
633 were regularly monitored to avoid fouling and overfilling due to the bivalve growth.  
634 After about one month of acclimation, the cohabiting oysters and mussels were sampled  
635 at low tide. The same work plan was followed in 2012 with the exception that diploid *C.*  
636 *gigas* spat was obtained from a local hatchery (Chioggia, Venice).

637 Eleven sampling campaigns were carried out on May 30th 2011, June 20th 2011, July  
638 18th 2011, August 1st 2011, October 10th 2011, February 14th 2012, May 21st 2012,  
639 June 18th 2012, July 16th 2012, September 3rd 2012, January 14th 2013. During each  
640 campaign water, sediment, zooplankton and bivalve (*M. galloprovincialis* and *C. gigas*)  
641 samples were collected. Water samples were collected into sterile bottles and filtered on

642 a piece of the 200- $\mu\text{m}$  net in order to remove the highly particulate matter (zooplankton  
643 included). Five hundred milliliters of the resulting water were then filtered onto a 0.22-  
644  $\mu\text{m}$ -pore-size Millipore membrane (47 mm in diameter) (Millipore, Milan, Italy). Filter  
645 bound material was suspended in ASW (50 ml) by vortexing and used for  
646 microbiological and molecular investigations. Surface sediment samples (0-2 cm) were  
647 collected by manual sediment core. For DNA extraction and direct bacterial  
648 quantification by Real-Time PCR, 1 g of sediment was used. For culture dependent  
649 assays, sediment aliquots (5 g) were suspended in 50 ml ASW, vigorously vortexed for  
650 30 s and treated in a sonication bath for 60 s (for a total of three times); supernatants  
651 were collected and processed. Zooplankton organisms ( $>200\ \mu\text{m}$ ) were collected by  
652 dragging the water horizontally, at a depth of about 1 m, with a 200- $\mu\text{m}$ -mesh plankton  
653 net. Five to 10  $\text{m}^3$  of water were dragged at each sampling. In the laboratory, the whole  
654 zooplankton suspension was passed through a 200  $\mu\text{m}$  mesh filter and total zooplankton  
655 was collected; after washing with ASW, 0.25 g of zooplankton were used for DNA  
656 extraction and bacterial quantification by Real-Time PCR. Another 1 g aliquot was  
657 suspended in ASW (50 ml), vigorously vortexed for 30 s and treated in a sonication  
658 bath for 60 s (for a total of three times) and used for culture dependent assays.

659

660 ***Detection, enumeration and isolation of culturable vibrios in environmental and***  
661 ***bivalve samples***

662 The most-probable-number technique was used to enumerate the number of culturable  
663 *Vibrio* spp., in the water, zooplankton and sediment samples. Three dilution series  
664 (1:10) were carried out in five replicate culture tubes, starting with 0.5 ml of prepared  
665 samples and 4.5 ml of Alkaline Peptone Water (APW, final pH  $8.6 \pm 0.2$ ) as the growth  
666 medium. Tubes were incubated at  $24^\circ\text{C}$  for 8 h in the dark and growth was assessed by  
667 plating each APW tube onto Thiosulfate Citrate Bile Sucrose (TCBS) (Conda  
668 laboratories, Madrid, Spain) plates. Plates were incubated at  $20^\circ\text{C}$  for 48 to 96h. For  
669 enumeration of *Vibrio* spp. in oysters and mussels, pools of 30 animals were  
670 homogenized in a Polytron PT 3000 Kinematica AG homogenizer and dilutions to the  
671 1/100 and 1/10.000 were performed in ASW. One hundred  $\mu\text{l}$  of those dilutions were  
672 plated on TCBS plates and incubated for 48 to 96 h at  $20^\circ\text{C}$ . Microorganisms were  
673 counted as *Vibrio* spp. if they formed 2-3 mm yellow or green colonies on TCBS  
674 medium, were Gram-negative and curved-bacillus-shaped, oxidase-positive and

675 fermentative with glucose by the oxidation/fermentation test. For the isolation and  
 676 identification of *V. splendidus* clade bacteria and *V. aestuarianus*, colonies were tested  
 677 by clade and species-specific Real Time PCR assays respectively, as previously  
 678 described. Bacterial isolates identified as belonging to *V. splendidus* clade were further  
 679 identified at the species level by sequencing the *pyrH* gene following the protocol  
 680 described by Tall *et al.*, (2013).

681

### 682 ***Detection and enumeration of vibrios in environmental and bivalve samples by Real-*** 683 ***Time PCR***

684 Nucleic acids from zooplankton, mussels (0.25 g) and oyster (0.25 g) samples were  
 685 extracted with the High Pure Polymerase Chain Reaction (PCR) Template Preparation  
 686 Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's  
 687 instructions. Nucleic acids from seawater and sediment samples were extracted as  
 688 previously described for *in vitro* experiments. For *Vibrio* spp. enumeration genus-  
 689 specific primers (F-GGCGTAAAGCGCATGCAGGT; R-  
 690 GAAATTCTACCCCTCTACAG, Thompson *et al.*, 2004) and the LightCycler-  
 691 FastStart DNA Master SYBR Green I kit optimised for use with glass capillaries were  
 692 used following conditions described in Vezzulli *et al* (2009). For the enumeration of *V.*  
 693 *splendidus* clade bacteria and *V. aestuarianus* a Taqman Real-Time PCR protocol was  
 694 performed as previously described.

695

### 696 ***Characterization of environmental isolates***

697 All *Vibrio* isolates belonging to *V. splendidus* clade and *V. aestuarianus* were also  
 698 analyzed for the presence of genes encoding for adhesins to chitin substrates (*mshA* and  
 699 *gbpA*) and genes encoding for virulence associated traits (*ompU* and *vsm/vam*). Primers  
 700 used were the following: mshavsF- ACTTACAAGGCGATGCTCGT and mshavsR-  
 701 TCCACGGCTTCTTCAATACC (*mshA* of *V. splendidus*, this study); mshavaF-  
 702 GCGGTTTCACCCTTATTGAA and mshavaR -ATACCAGCGGCACCATCTAA  
 703 (*mshA* of *V. aestuarianus*, this study); gbpAvaF-TATTACTCCCGCGCAAATC and  
 704 gbpAvaR-ATGCCATAGAGCGGAGAGAA (*gbpA* of *V. aestuarianus*, this study);  
 705 gbpAF-TGTCGTTGAAGGAAATATGGTG and gbpA-R  
 706 TCTTTCAGATAAATCGGGTTGG (*gbpA* of *V. splendidus*-clade, Stauder *et al.*,  
 707 2010); 2494-5 TGACCGTGCTGACAACATGC and 2494-6

708 TTAGAAGTCGTAACGTAGACC (*ompU* of *V. splendidus* and *V. aestuarianus*,  
709 Duperthuy *et al.*, 2010); *vsmF*-TCCAACAGAGCCTCGTCG and *vsmR*-  
710 AAGGGCGTTCTGGCATAG (*vsm* of *V. splendidus*, Liu *et al.*, 2013); *vamF*-  
711 TTGACGTGGTTGATGCATTT and *vamR*-AGATCGGCATCACCTGTACC (*vam* of  
712 *V. aestuarianus*, this study)

713

#### 714 ***Environmental variables***

715 Daily records of SST (°C) and salinity values (PSU) were measured in the Goro lagoon  
716 from May 2011 to January 2013 by means of a CTD-multiprobe.

717

#### 718 ***Statistical tests***

719 One-way unpaired T -test was used to compare average values of adhesion and biofilm  
720 formation among bacterial strains. Pearson correlation analysis was performed to  
721 investigate the relationship between bacterial and environmental variables.

722

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935 **Table 1**

936 Attachment of *V. splendidus* LGP32, *V. aestuarianus* 01/032 and *V. cholerae* O1 14034  
 937 strains to chitin particles and to the copepod *T. fulvus*. Values are means of three assays  
 938 performed on the same day with the same bacterial suspension. Percentages of  
 939 inhibition in comparison with untreated control are reported in brackets; the symbol –  
 940 means no inhibition, nd means not determined

941

Strain	Experimental conditions	Attachment to chitin (bacteria per 2,5 mg of chitin particles)	Attachment to copepods (bacteria per copepod)
<i>V. aestuarianus</i> 01/032	control	$6.0 \times 10^4 \pm 5 \times 10^3$	$3.3 \times 10^2 \pm 4 \times 10^1$
	+GlcNac	$2.3 \times 10^4 \pm 1 \times 10^3$ (62% inhibition)	nd
	GlcNac pretreatment	$2.9 \times 10^4 \pm 4 \times 10^3$ (51% inhibition)	nd
	+D-mannose	$2.7 \times 10^4 \pm 3 \times 10^3$ (55% inhibition)	nd
	D-mannose pretreatment	$3.4 \times 10^4 \pm 5 \times 10^3$ (43% inhibition)	nd
<i>V. splendidus</i> LGP32	control	$1.2 \times 10^5 \pm 2 \times 10^4$	$2.4 \times 10^3 \pm 3 \times 10^2$
	+GlcNac	$1.4 \times 10^5 \pm 1 \times 10^4$ (–)	nd
	GlcNac pretreatment	$1.5 \times 10^5 \pm 2 \times 10^4$ (–)	nd
	+D-mannose	$1.6 \times 10^5 \pm 2 \times 10^4$ (–)	nd
	D-mannose pretreatment	$1.5 \times 10^5 \pm 1 \times 10^4$ (–)	nd
<i>V. cholerae</i> O1 14034	control	$9.1 \times 10^6 \pm 0.9 \times 10^6$	$5.0 \times 10^4 \pm 7 \times 10^3$

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946 **Figure captions**

947

948 **Figure 1**

949 Temporal variation in the percentage of total (black circle), viable (black square) and  
950 culturable (black triangle) *V. splendidus* LGP32 and *V. aestuarianus* 01/32 cells  
951 incubated in seawater under aseptic conditions and at different temperature, salinity and  
952 nutrient concentration: 25°C, 20‰ salinity (a); 25°C, 35‰ salinity (b); 5°C, 20‰  
953 salinity (c), 5°C, 35‰ salinity (d); 25°C, 20‰ salinity, 40mg/L Peptone (e). Results are  
954 the average of three replicate experiments

955

956 **Figure 2**

957 Temporal variation in the percentage of total (black circle), viable (black square) and  
958 culturable (black triangle) *V. splendidus* LGP32 and *V. aestuarianus* 01/32 cells  
959 incubated in the sediment under aseptic conditions at 5°C, 20‰ salinity. Results are the  
960 average of three replicate experiments

961

962 **Figure 3**

963 Biofilm formation of *V. splendidus* LGP32 and *V. aestuarianus* 01/32 strains on PVC  
964 surfaces at 18°C (black square) and 24°C (black triangle). Results are the average of  
965 three replicate experiments

966

967 **Figure 4**

968 Percentage of live (L) and dead (D) cells of *V. splendidus* LGP32 and *V. aestuarianus*  
969 01/32 strains calculated on total, >0.2µm and <0.2µm cell size fractions after prolonged  
970 exposure (90 days) to ASW (20‰ salinity) at 5 °C under starving condition. The  
971 number of total cells (total) at the beginning of the experiment and the number of  
972 culturable cells (culturable fraction) following incubation are also reported

973

974 **Figure 5**

975 Standard microscopic imaging (magnification 1000X) showing morphological changes  
976 observed in *V. aestuarianus* 01/32 cells following prolonged exposure to ASW (20‰  
977 salinity) at 5 °C under starving condition. Similar results were obtained for *V.*  
978 *splendidus* LGP32 strains



979

980 **Figure 6**

981 BIVALIFE experimental station in Goro lagoon (Northern Adriatic Sea, Italy)

982

983 **Figure 7**

984 Temporal occurrence of *Vibrio* spp. enumerated by culture-based (light grey bar) and  
985 molecular (dark grey bar) methods in environmental matrices and bivalves collected at  
986 the BIVALIFE experimental station in the Goro lagoon

987

988 **Figure 8**

989 Average Real-Time PCR counts of *V. splendidus* clade bacteria (a) and *V. aestuarianus*  
990 (c) in warm and cold months in the different environmental matrices and bivalves in the  
991 Goro lagoon during the study period (May 2011 to January 2013). Months when the  
992 bacteria were detected are superimposed on the histogram bars

993

994 **Figure 9**

995 Presence and distribution of genes linked to environmental persistence and virulence in  
996 environmental isolates of *V. splendidus*, *V. aestuarianus* and other *V. splendidus*-clade  
997 bacterial species isolated from the Goro lagoon

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1010 **Supplementary Material**

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1012 **Supplementary Table 1**

1013 *Vibrio* strains isolated from the different environmental matrices and bivalves in the  
 1014 Goro lagoon during the study period (May 2011 to January 2013). Isolates belonging to  
 1015 the species *V. splendidus* and *V. aestuarianus* are indicated in bold. Presence (+) and  
 1016 absence (-) of genes encoding for adhesins to chitin substrates (*mshA* and *gbpA*) and  
 1017 genes encoding for virulence associated traits (*ompU* and *vsm/vam*) is also reported

1018

Isolate	Matrix	Date	Species	<i>mshA</i>	<i>gbpA</i>	<i>ompU</i>	<i>vsm/vam</i>
			<b><i>V. splendidus</i></b>	+	-	+	+
			<b>LGP32</b>				
			<b><i>V. aestuarianus</i></b>	+	+	+	+
			<b>01/32</b>				
<b>165</b>	<b>Oyster</b>	<b>14/02/2012</b>	<b><i>V. splendidus</i></b>	-	-	-	-
<b>168</b>	<b>Oyster</b>	<b>21/05/2012</b>	<b><i>V. splendidus</i></b>	-	-	+	+
207A	Oyster	18/06/2012	<i>V. celticus</i>	-	-	+	-
207B	Oyster	18/06/2012	<i>V. celticus</i>	-	-	+	-
<b>162</b>	<b>Mussel</b>	<b>14/02/2012</b>	<b><i>V. splendidus</i></b>	-	-	-	-
173	Mussel	21/05/2012	<i>V. crassostreae</i>	-	+	-	+
174	Mussel	21/05/2012	<i>V. celticus</i>	-	-	+	-
202	Mussel	18/06/2012	<i>V. celticus</i>	-	-	+	+
<b>334</b>	<b>Mussel</b>	<b>14/01/2013</b>	<b><i>V. splendidus</i></b>	-	-	+	+
19	Zooplankton	30/05/2011	<i>V. celticus</i>	-	-	+	-
<b>156</b>	<b>Zooplankton</b>	<b>14/02/2012</b>	<b><i>V. splendidus</i></b>	-	-	+	-
179bis	Zooplankton	21/05/2012	<i>V. chagasii</i>	-	-	-	+
<b>180</b>	<b>Zooplankton</b>	<b>21/05/2012</b>	<b><i>V. splendidus</i></b>	-	-	+	+
<b>152</b>	<b>Sediment</b>	<b>14/02/2012</b>	<b><i>V. splendidus</i></b>	-	-	+	+
<b>153</b>	<b>Sediment</b>	<b>14/02/2012</b>	<b><i>V. splendidus</i></b>	-	-	+	-
190	Sediment	21/05/2012	<i>V. celticus</i>	-	-	+	-
<b>190bis</b>	<b>Sediment</b>	<b>21/05/2012</b>	<b><i>V. splendidus</i></b>	-	-	+	+
<b>325</b>	<b>Sediment</b>	<b>14/01/2013</b>	<b><i>V. splendidus</i></b>	-	-	+	-
2	Seawater	30/05/2011	<i>V. chagasii</i>	-	+	-	+
8	Seawater	30/05/2011	<i>V. celticus</i>	-	-	-	+
<b>147</b>	<b>Seawater</b>	<b>14/02/2012</b>	<b><i>V. splendidus</i></b>	-	-	+	+
150	Seawater	14/02/2012	<i>V. lentus</i>	-	-	+	-
151	Seawater	14/02/2012	<i>V. lentus</i>	-	-	-	-
184	Seawater	21/05/2012	<i>V. chagasii</i>	-	-	-	+
<b>185</b>	<b>Seawater</b>	<b>21/05/2012</b>	<b><i>V. splendidus</i></b>	-	-	+	+
<b>279</b>	<b>Seawater</b>	<b>03/09/2012</b>	<b><i>V. splendidus</i></b>	-	-	-	-
318	Seawater	14/01/2013	<i>V. splendidus</i>	-	-	+	-
<b>36</b>	<b>Oyster</b>	<b>30/05/2011</b>	<b><i>V. aestuarianus</i></b>	+	+	+	-
<b>38</b>	<b>Oyster</b>	<b>30/05/2011</b>	<b><i>V. aestuarianus</i></b>	-	+	+	+
<b>40</b>	<b>Oyster</b>	<b>30/05/2011</b>	<b><i>V. aestuarianus</i></b>	+	+	+	+
<b>29</b>	<b>Mussel</b>	<b>30/05/2011</b>	<b><i>V. aestuarianus</i></b>	-	+	-	+

32	Mussel	30/05/2011	<i>V. aestuarianus</i>	-	-	+	+
33	Mussel	30/05/2011	<i>V. aestuarianus</i>	-	-	+	+
17	Zooplankton	30/05/2011	<i>V. aestuarianus</i>	+	+	+	+
18	Zooplankton	30/05/2011	<i>V. aestuarianus</i>	-	+	-	+
241	Zooplankton	16/07/2012	<i>V. aestuarianus</i>	-	+	+	+
244	Zooplankton	16/07/2012	<i>V. aestuarianus</i>	-	+	+	+
245	Zooplankton	16/07/2012	<i>V. aestuarianus</i>	-	+	+	+
287	Sediment	03/09/2012	<i>V. aestuarianus</i>	-	+	+	+

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1020 **Supplementary Figure 1**

1021 Scatter plots showing relationships existing between *V. splendidus* clade bacteria, *V.*  
1022 *aestuarianus*, and *Vibrio* spp. in the different environmental matrices and bivalves and  
1023 sea surface temperature values in the Goro lagoon during the study period (May 2011 to  
1024 January 2013). Grey backgrounds behind plots indicate a significant correlation  
1025 ( $p < 0.05$ ) between variables.

1026

1027 **Supplementary Figure 2**

1028 Scatter plots showing relationships existing between *V. splendidus* clade bacteria, *V.*  
1029 *aestuarianus*, and *Vibrio* spp. in the different environmental matrices and bivalves and  
1030 salinity values in the Goro lagoon during the study period (May 2011 to January 2013).  
1031 Grey backgrounds behind plots indicate a significant correlation ( $p < 0.05$ ) between  
1032 variables.

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*V. splendidus* LGP32    *V. aestuarianus* 01/32

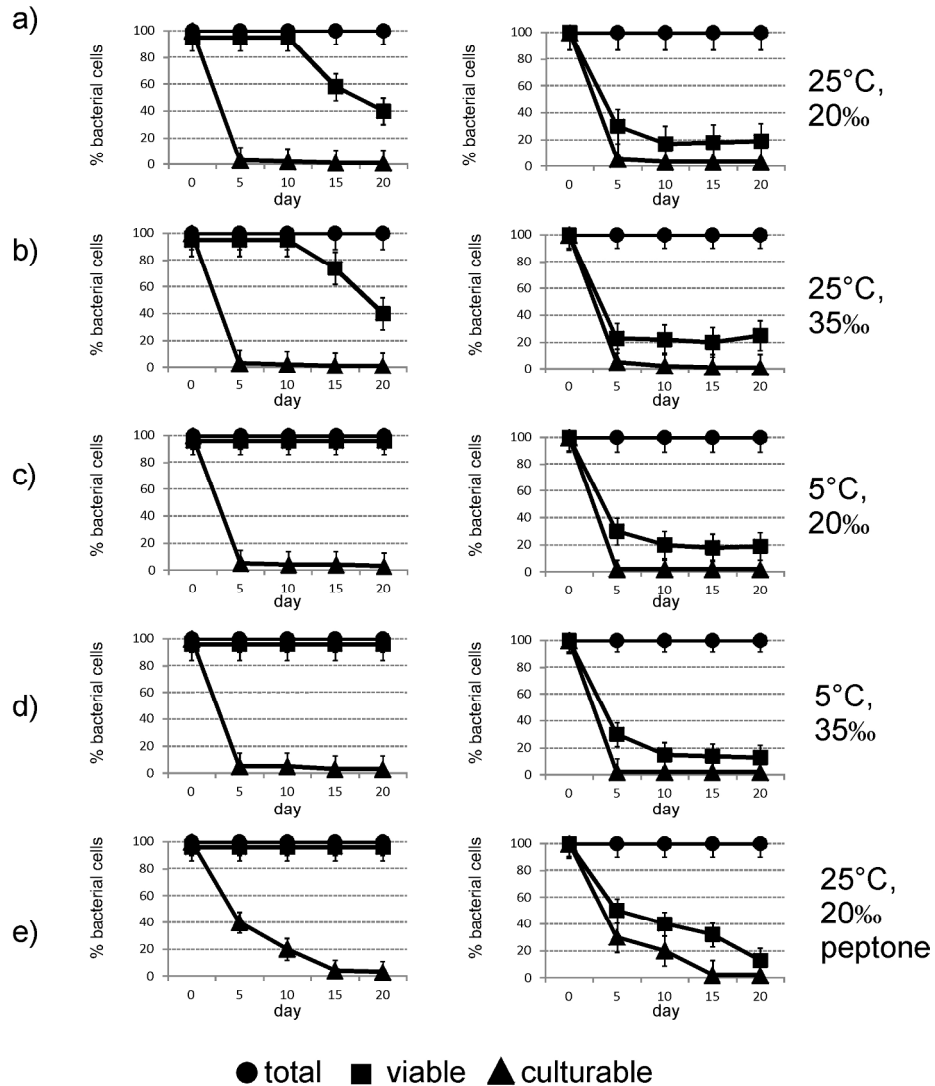


Figure 1

Temporal variation in the percentage of total (black circle), viable (black square) and culturable (black triangle) *V. splendidus* LGP32 and *V. aestuarianus* 01/32 cells incubated in seawater under aseptic conditions and at different temperature, salinity and nutrient concentration: 25°C, 20‰ salinity (a); 25°C, 35‰ salinity (b); 5°C, 20‰ salinity (c), 5°C, 35‰ salinity (d); 25°C, 20‰ salinity, 40mg/L Peptone (e). Results are the average of three replicate experiments

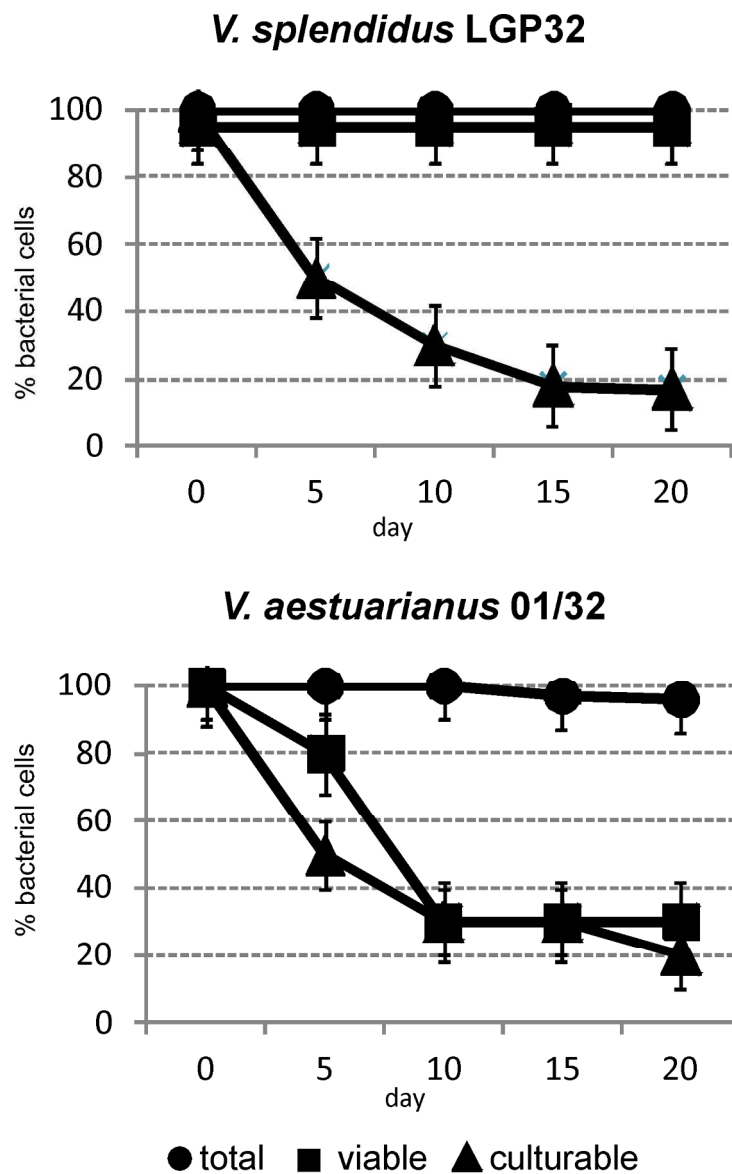


Figure 2  
Temporal variation in the percentage of total (black circle), viable (black square) and culturable (black triangle) *V. splendidus* LGP32 and *V. aestuarianus* 01/32 cells incubated in the sediment under aseptic conditions at 5°C, 20‰ salinity. Results are the average of three replicate experiments

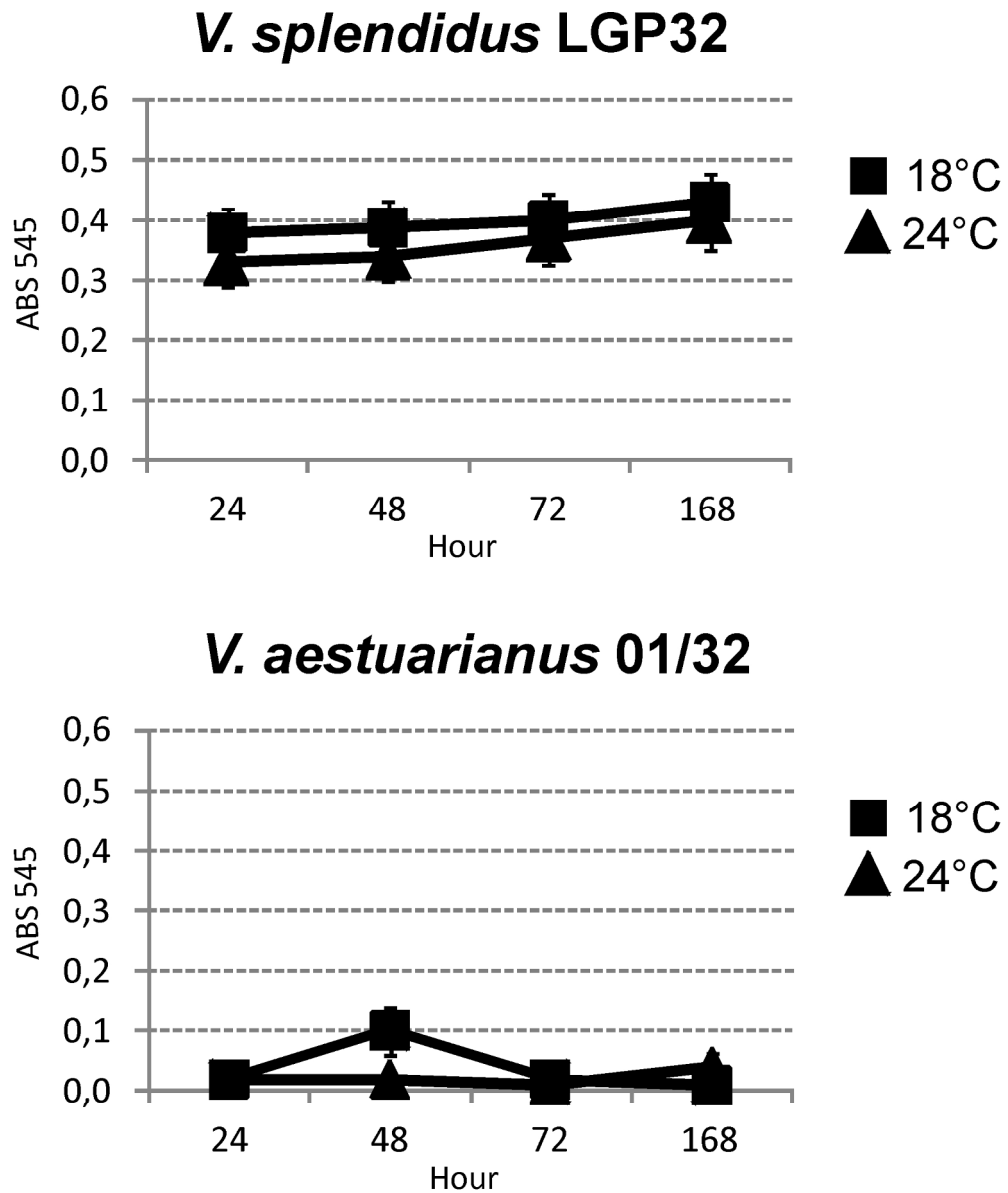


Figure 3

Biofilm formation of *V. splendidus* LGP32 and *V. aestuarianus* 01/32 strains on PVC surfaces at 18°C (black square) and 24°C (black triangle). Results are the average of three replicate experiments

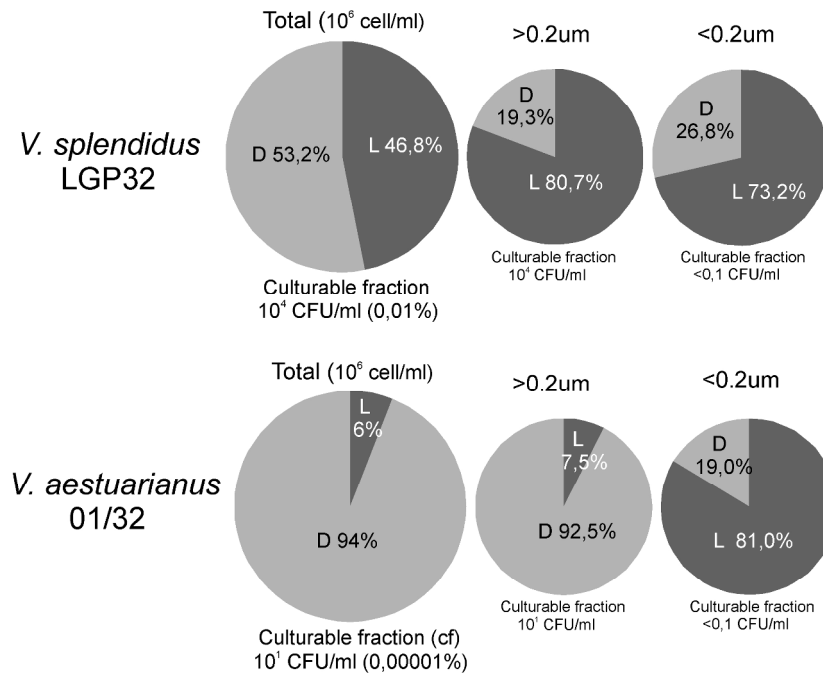


Figure 4

Percentage of live (L) and dead (D) cells of *V. splendidus* LGP32 and *V. aestuarianus* 01/32 strains calculated on total, >0.2µm and <0.2µm cell size fractions after prolonged exposure (90 days) to ASW (20‰ salinity) at 5 °C under starving condition. The number of total cells (total) at the beginning of the experiment and the number of culturable cells (culturable fraction) following incubation are also reported

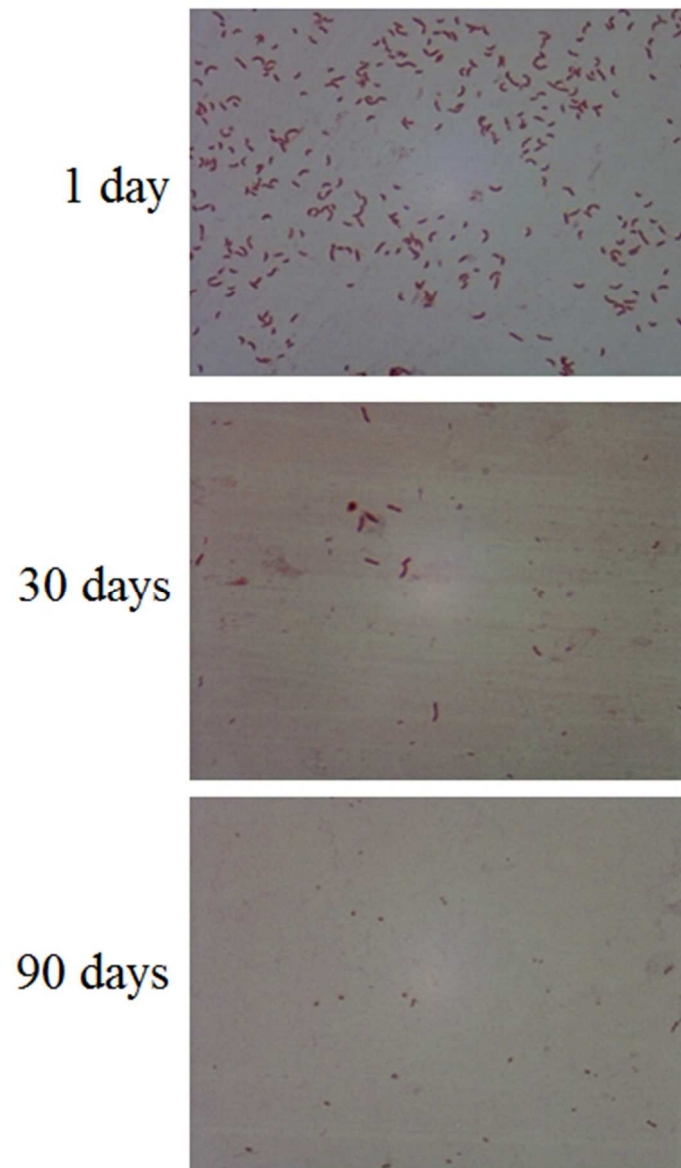


Figure 5  
Standard microscopic imaging (magnification 1000X) showing morphological changes observed in *V. aestuarianus* 01/32 cells following prolonged exposure to ASW (20‰ salinity) at 5 °C under starving condition. Similar results were obtained for *V. splendidus* LGP32 strains

104x175mm (96 x 96 DPI)



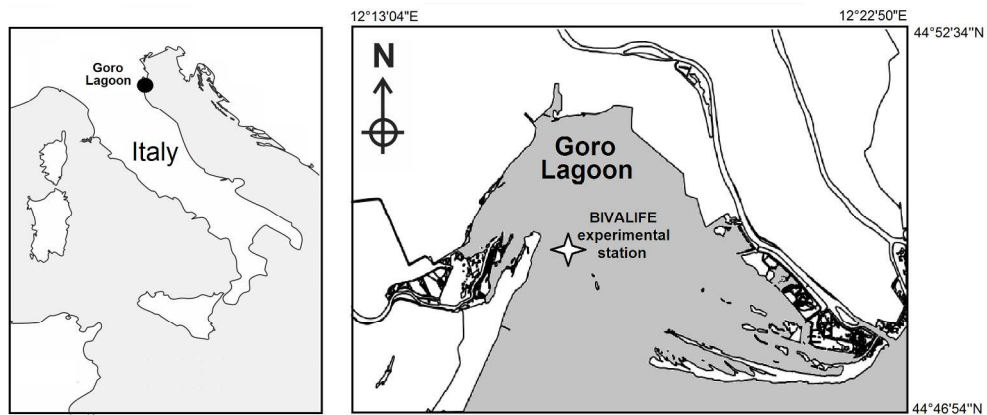


Figure 6  
BIVALIFE experimental station in Goro lagoon (Northern Adriatic Sea, Italy)

657x279mm (96 x 96 DPI)

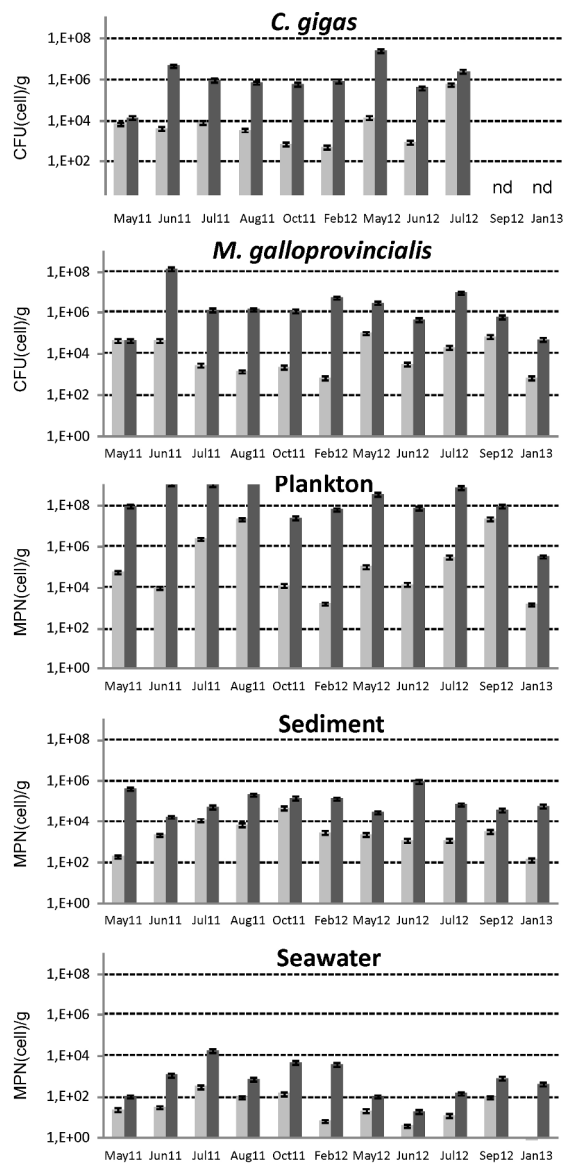


Figure 7  
 Temporal occurrence of *Vibrio* spp. enumerated by culture-based (light grey bar) and molecular (dark grey bar) methods in environmental matrices and bivalves collected at the BIVALIFE experimental station in the Goro lagoon

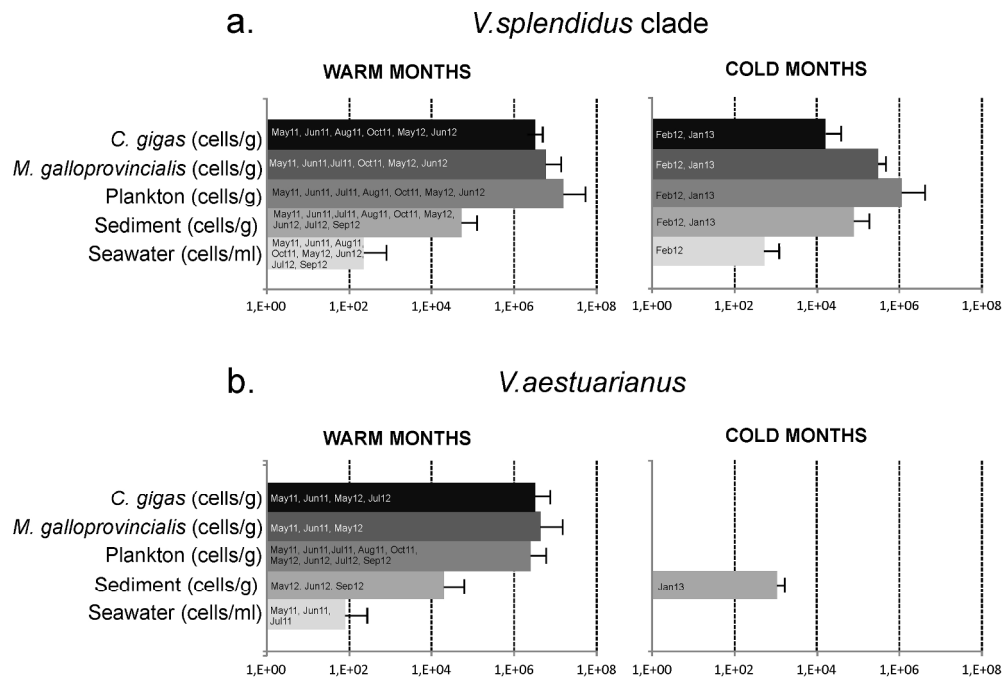


Figure 8

Average Real-Time PCR counts of *V. splendidus* clade bacteria (a) and *V. aestuarianus* (c) in warm and cold months in the different environmental matrices and bivalves in the Goro lagoon during the study period (May 2011 to January 2013). Months when the bacteria were detected are superimposed on the histogram bars

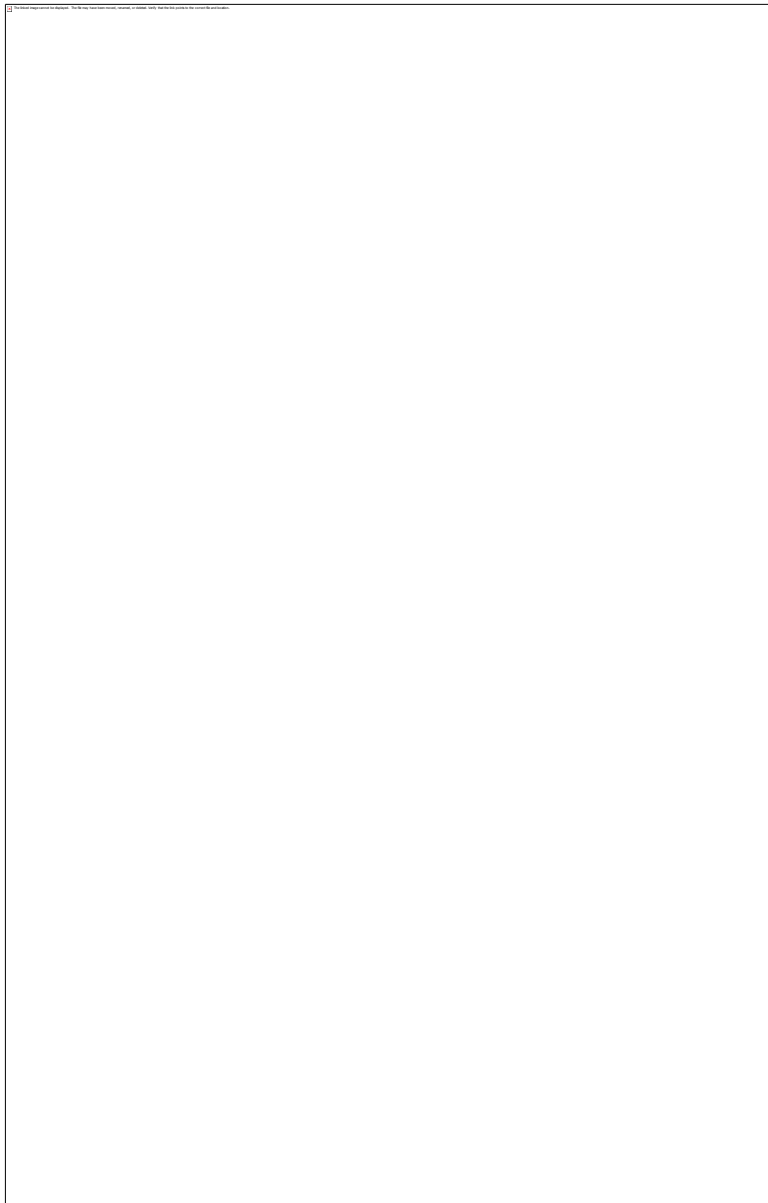


Figure 9  
Presence and distribution of genes linked to environmental persistence and virulence in environmental isolates of *V. splendidus*, *V. aestuarianus* and other *V. splendidus*-clade bacterial species isolated from the Goro lagoon