environmental microbiology
environmental microbiology reports

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Journal:	*Environmental Microbiology and Environmental Microbiology Reports*
Manuscript ID:	EMI-2014-0153.R1
Manuscript Type:	EMI - Research article
Journal:	Environmental Microbiology
Date Submitted by the Author:	n/a
Complete List of Authors:	Vezzulli, Luigi; University of Genoa, DISTAV Pezzati, Elisabetta; University of Genoa, DISTAV Stauder, Monica; University of Genoa, DISTAV Stagnaro, Laura; University of Genoa, DISTAV Venier, Paola Pruzzo, Carla; Universita degli Studi di Genova, Dipartimento di Biologia
Keywords:	microbial ecology, pathogen ecology, bacteria, adhesion

Published in Environmental Microbiology (2015)17(4), 1065–1080
doi:10.1111/1462-2920.12484

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

****REVISED****

Luigi Vezzulli[1+], Elisabetta Pezzati[1+], Monica Stauder[1], Laura Stagnaro[1], Paola Venier[2], Carla Pruzzo[1]*

[1]Department of Life, Earth and Environmental Sciences (DISTAV), University of Genoa, Genoa, Italy
[2] Department of Biology, University of Padova, Padova, Italy

+ these authors contributed equally to this work

*Correspondence: Carla Pruzzo, Department of Life, Earth and Environmental Sciences (DISTAV), University of Genoa, Corso Europa, 26, Genoa 16132, Italy. Phone: +39-010-3538251; fax: +39-010-3538267; e-mail: carla.pruzzo@unige.it

Journal: Environmental Microbiology

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

Abstract

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

Introduction

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

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

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

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

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

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

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

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

Results and Discussion

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

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

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

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

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

Survival strategies in the aquatic environment: adhesion to environmental chitin surfaces

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

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

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

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

Survival strategies in the aquatic environment: biofilm formation

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

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

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

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

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

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

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

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

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

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

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

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

to the other environmental matrices, with values generally exceeding 10^6 cells/g both in warm and cold months (**Fig. 8**). These bacteria were also frequently detected in oysters and mussels; when present, their concentration ranged from $1.1x10^5$ to $1.5x10^7$ cells/g and from $2.8x10^2$ to $3.8x10^7$ cells/g for oyster and mussel respectively.

In seawater and sediment compartments, when present, their concentration ranged from $1.9x10^1$ to $7.6x10^2$ cells/ml and from $7.4x10^2$ to $1.6x10^5$ cells/g, respectively. The sediment was the compartment where *V. splendidus*-clade bacteria were more frequently found during the study period (9 out of 11 sampling dates) and may thus represents a suitable niche for their persistence in the environment. *V. splendidus*-clade bacteria were also found associated to a variety of other environmental substrates commonly found in the lagoon including large benthic crustaceans (*Dyspanopeus sayi*, *Upogebia pusilla*, *Cratigon* sp., *Carcinus aestuarii*), *Gracilaria verrucosa* macroalgae and *Tapes philippinarum* clam (data not shown).

In contrast to *V. splendidus*, *V. aestuarianus* cells were rarely detected in the Goro lagoon samples. In particular, high concentrations of *V. aestuarianus* bacteria were found associated to zooplankton during warm months, with values ranging from $1.4x10^5$ to $6.9x10^6$ cells/g, and only sporadically found in mussels, oysters, seawater and sediments (**Fig. 8**). In particular, during cold months, *V. aestuarianus* was detected only once (January 2013) in the sediment compartment. The sediment may thus represent an environmental reservoir also for this species where, in accordance with results from laboratory experiments (**Fig. 2**), the bacteria can find a favorable environment for overwintering (Vezzulli *et al*., 2009).

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

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

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

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

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

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

Regarding genes encoding for adhesins involved in attachment to chitin, all *V. splendidus* isolates lacked both *mshA* and *gbpA*. In contrast, almost all *V. aestuarianus* 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 s*trains 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

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

Experimental procedures

In vitro studies

Bacterial strains and culture conditions

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

Setting of experimental microcosms for "survival" and "VBNC induction" experiments

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

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

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

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

Nucleic acid extraction

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

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

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

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

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

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

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

Enumeration of total and viable bacterial cells by Epifluorescence microscopy

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

Enumeration of culturable bacterial cells

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

Adhesion assays

To assess bacterial attachment to chitin particles one volume bacterial suspension ($5x10^7$ cell/ml) was added to UV-sterilized chitin purified from crab shell (2.5 mg;

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

To assess bacterial attachment to copepods a similar protocol was followed. *T. fulvus* copepods belonging to the *Harpacticoida* family were cultured in filter-sterilized ASW and fed with unicellular phytoplanktonic algae (*Tetraselmis suecica*). Before the association assay, the copepods were vigorously washed 10 times with ASW to remove most adhering bacteria and heat killed (65°C per 15 min). One ml of bacterial suspension ($1x10^8$ cell/ml) was then added to 10 washed copepods in a tube and incubated at room temperature over night. After overnight incubation, copepods were collected and gently washed three times with phosphate buffered saline (PBS) solution (137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) PBS to remove non-adherent bacteria. 200 ul of PBS was then added to the washed copepods and DNA was extracted as previously described. The number of attached bacteria were determined by Real Time PCR. All adhesion assays were performed in triplicate.

Biofilm assays

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

200 µl of ethanol : acetone (8:2) per well. After about 1 h incubation, biofilm formation was quantified by measuring the absorbance (Abs) of the solution at 545~~0~~ nm.

For the purposes of comparative analysis, we classified bacteria into three arbitrarily defined categories based upon $Abs_{545~~0~~}$ of the supernatant as follow: $Abs_{545~~0~~} \leq 0.150$ = non-producer~~adherent bacteria~~; $Abs_{545~~0~~} \geq 0.250$ = strong~~ly~~ ~~adherent~~producer; $0.150 < Abs_{545~~0~~} < 0.250$ = weak producer~~ly adherent~~. Tests were done in triplicate on three separate occasions and the results averaged.

In field studies

Experimental design and field sampling

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

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

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

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

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

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

The most-probable-number technique was used to enumerate the number of culturable *Vibrio* spp., in the water, zooplankton and sediment samples. Three dilution series (1:10) were carried out in five replicate culture tubes, starting with 0.5 ml of prepared samples and 4.5 ml of Alkaline Peptone Water (APW, final pH 8.6± 0.2) as the growth medium. Tubes were incubated at 24°C for 8 h in the dark and growth was assessed by plating each APW tube onto Thiosulfate Citrate Bile Sucrose (TCBS) (Conda laboratories, Madrid, Spain) plates. Plates were incubated at 20°C for 48 to 96h. For enumeration of *Vibrio* spp. in oysters and mussels, pools of 30 animals were homogenized in a Polytron PT 3000 Kinematica AG homogenizer and dilutions to the 1/100 and 1/10.000 were performed in ASW. One hundred µl of those dilutions were 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 GAAATTCTACCCCCCTCTACAG, 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 GCGGTTTCACCCTTATTGAA and mshavaR –ATACCAGCGGCACCATCTAA
704 (*mshA* of *V. aestuarianus*, this study); gbpAvaF-TATTACTCCCGCGCAAAATC and
705 gbpAvaR-ATGCCATAGAGCGGAGAGAA (*gbpA* of *V. aestuarianus*, this study);
706 gbpAF-TGTCGTTGAAGGAAATATGGTG and gbpA-R

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

Environmental variables

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

Statistical tests

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

Acknowledgements

The authors would like to thank Stefano Gualdi (University of Genoa, Italy) for his assistance in the conduction of microcosm experiments, Prof. Luigi Pane (DISTAV, University of Genoa, Italy) for kindly providing *T. fulvus* copepods and Dr. Edoardo Turolla (Istituto Delta, Goro, Italy) for technical help in the establishment of the experimental station in the Goro lagoon and support during field sampling. Authors of this manuscript have no conflict of interest to declare. This work was promoted and funded by the FP7-European project BIVALIFE "Controlling infectious diseases in oysters and mussels in Europe" (Grant Agreement 266157)

References

Balbi, T., Fabbri, R., Cortese, K., Smerilli, A., Ciacci, C., Grande., C. *et al.* (2013) Interactions between Mytilus galloprovincialis hemocytes and the bivalve pathogens *Vibrio aestuarianus* 01/032 and *Vibrio splendidus* LGP32 (2013) Fish Shellfish Immun 35(6):1906 -1915.

Binesse, J., Delsert, C., Saulnier, D., Champomier-Verge`s, M.C., Zagorec, M., Munier-Lehmann, H., *et al* (2008) Metalloprotease *vsm* is the major determinant of toxicity for extracellular products of *Vibrio splendidus*. *Appl Environ Microbiol* **74**:7108–7117.

Chiavelli, D.A., Marsh, J.W., and Taylor, R.K. (2001) The mannose-sensitive hemagglutinin of *Vibrio cholerae* promotes adherence to zooplankton. *Appl Environ Microbiol* **67**:3220-3225.

Duperthuy, M., Binesse, J.D., Le Roux, F., Romestand, B., Caro, A., Got, P., *et al*. (2010) The major outer membrane protein OmpU of *Vibrio splendidus* contributes to host antimicrobial peptide resistance and is required for virulence in the oyster *Crassostrea gigas*. *Environ Microbiol* **12**(4): 951-963.

Duperthuy. M,, Schmitta, P., Garzónd, E., Caro, A., Rosa, R.D., Le Rouxe, F., *et al* (2011) Use of OmpU porins for attachment and invasion of *Crassostrea gigas* immune cells by the oyster pathogen *Vibrio splendidus*. *P Natl Acad Sci Usa* **108**: 2993-2998.

FAO 2013 Global Aquaculture Production Statistics for the year 2011 (http://www.fao.org/figis/servlet/TabLandArea?tb_ds=Aquaculture&tb_mode=TABLE &tb_act=SELECT&tb_grp=COUNTRY)

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

Gay, M., Renault, T., Pons, A.M., and Le Roux, F. (2004b). Two *Vibrio splendidus* related strains collaborate to kill *Crassostrea gigas*: taxonomy and host alterations. *Dis Aquat Organ* **62**:65–74.

Garnier, M., Labreuche, Y., and Nicolas, J.L. (2008) Molecular and phenotypic characterization of *Vibrio aestuarianus* subsp. *francensis* subsp. nov., a pathogen of the oyster *Crassostrea gigas*. *Syst Appl Microbiol* **31**:358e65.

Hall-Stoodley, L., and Stoodley, P. (2005) Biofilm formation and dispersal and the transmission of human pathogens. *Trends Microbiol* **13**: 7–10.

Hobbie, J.E., Daley, R.J., and Jasper, S., (1977). Use of Nuclepore filters for counting bacteria by fluorescence microscopy. *Appl Environ Microbiol* **33**:1225–1228.

Huq, A., Whitehouse, C.A., Grim, C.J., Alam, M., Colwell, R.R. (2008) Biofilms in water, its role and impact in human disease transmission. *Curr Opin Biotech* **19**: 244-247.

IFREMER report (2013) *Vibrio splendidus* et *V. aestuarianus* detection by Real Time Polymerase Chain Reaction European Union Reference Laboratory for Molluscs Diseases. Edition n°1, Laboratoire de Génétique et Pathologie des Mollusques Marins, Av. de Mus de Loup, 17390 La Tremblade France http://www.eurlmollusc.eu/content/download/72924/948279/file/Vsplendidus&aestuarianus%20_RealTimePCR.pdf

Johnson, C.N., Flowers, A.R., Noriea III, N.F., Zimmerman, A.M., Bowers, J.C., DePaola, A., *et al*. (2010) Relationships between environmental factors and pathogenic vibrios in the northern gulf of Mexico. *Appl Environ Microbiol* **76**(21): 7076-7084.

Labreuche, Y., Le Roux, F., Henry, J., Zatylny, C., Huvet, A., Lambert, C., *et al* (2010). *Vibrio aestuarianus* zinc metalloprotease causes lethality in the Pacific oyster *Crassostrea gigas* and impairs the host cellular immune defenses. *Fish Shellfish Immun* **29**: 753-758.

Lacoste, A., Jalabert, F., Malham, S., Cueff, A., Gélébart, F., Cordevant, C., Lange, M., and Poulet, S.A. (2001) A *Vibrio splendidus* strain is associated with summer mortality of juvenile oysters *Crassostrea gigas* in the Bay of Morlaix (North Brittany, France). *Dis Aquat Org* 46:139–145.

Le Roux, F., Gay, M., Lambert, C., Waechter, M., Poubalanne, S., Chollet, B., Nicolas, J.L., and Berthe, F.C.J. (2002) Comparative analysis of *Vibrio splendidus*-related strains isolated during *Crassostrea gigas* mortality events. *Aquat Living Resour* **15**:251–258.

Le Roux, F., Binesse, J., Saulnier, D., Mazel, D., (2007) Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene *vsm* by use of a novel counterselectable suicide vector. *Appl Environ Microbiol* **73**:777e84.

Liu, R., Qiu, L., Yu, Z., Zi, J., Yue, F., Wang, L., *et al* (2013) Identification and characterization of pathogenic *Vibrio splendidus* from Yesso scallop (*Patinopecten yessoensis*) cultured in a low temperature environment. *J Invertebr Pathol* **114(2)**:144-150.

Luna, G.M., Dell'Anno, A., and Danovaro, R., (2006) DNA extraction procedure: a critical issue for bacterial diversity assessment in marine sediments. *Environ Microbiol* **8**:308-320.

Matz, C., McDougald, D., Moreno, A.M., Yung, P.Y., Yildiz, F.H., Kjelleberg, S., (2005) Biofilm formation and phenotypic variation enhance predation-driven persistence of *Vibrio cholerae*. *Proc Natl Acad Sci USA* **102(46)**:16819-16824.

Meibom, K.L., Li, X.B., Nielsen, A.T., Wu, C.Y., Roseman, S., and Schoolnik, G.K. (2004) The *Vibrio cholerae* chitin utilization program. *Proc Natl Acad Sci USA* **101**: 2524–2529.

Moreno, M., Vezzulli, L., Marin, V., Laconi, P., Albertelli, G., and Fabiano, M., (2008) The use of meiofauna diversity as indicators of pollution in harbours. *ICES J Mar Sci* **65**: 1428-1435.

Moreno, M., Semprucci, F., Vezzulli, L., Balsamo, M., Fabiano, M., and Albertelli, G., (2011). The use of nematodes in assessing ecological quality status in the Mediterranean coastal ecosystems. *Ecol Indic* **61**: 417-421.

Oliver, J.D. (2005). The viable but nonculturable state in bacteria. **43**: 93-100.

Oliver, J.D. (2010). Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol Rev* **34**:415-425.

Oliver, J.D., Pruzzo, C., Vezzulli, L., Kaper, J.B., (2013) *Vibrio* Species," Food Microbiology: Fundamentals and Frontiers, 4th Ed. Edited by M. P. Doyle and R. L. Buchanan 2013 ASM Press, Washington, D.C. doi:10.1128/9781555818463.ch16

Pernet, F., Barret, J., Le Gall, P., Corporeau, C., Dégremont, L., Lagarde, F., *et al*. (2012) Mass mortalities of Pacific oysters *Crassostrea gigas* reflect infectious diseases and vary with farming practices in the Mediterranean Thau lagoon, France. *Aquacult Environ Interact* **2**: 215–237.

Pruzzo, C., Tarsi, R., Lleò, M.M., Signoretto, C., Zampini, M., Pane, L., *et al.* (2003) Persistence of adhesive properties in *Vibrio cholerae* after long-term exposure to sea water. *Environ Microbiol* **5(10)**:850-858.

Pruzzo, C., Vezzulli, L., and Colwell, R.R., (2008) Global impact of *Vibrio cholerae* interactions with chitin. *Environ Microbiol* **10(6)**:1400–1410.

Samain, J.F., and McCombie, H. (2008). Summer mortality of Pacific oyster *Crassostrea gigas*. The Morest Project. Versailles: Quae Editions. 379 pp. SAS Institute Inc. 1995. Basis statistics using SAS/STAT, software course notes. Cary, NC: SAS Institute. 720 pp.

Saulnier, D., De Decker, S., Haffner, P., Cobret, L., Robert, M., and Garcia, C. (2010) A Large-Scale Epidemiological Study to Identify Bacteria Pathogenic to Pacific Oyster *Crassostrea gigas* and correlation between virulence and metalloprotease-like activity. *Microb Ecol* **59**:787-798.

Sechi, L.A., Duprè, I., Deriu, A., Fadda, G., and Zanetti, S. (2000) Distribution of *Vibrio cholerae* virulence genes among different Vibrio species isolated in Sardinia, Italy. *J Appl Microbiol* **88**: 475–481.

Stauder, M., Vezzulli, L., Pezzati, E., Repetto, B., and Pruzzo C (2010) Temperature affects *Vibrio cholerae* O1 El Tor persistence in the aquatic environment via an enhanced expression of GbpA and MSHA adhesins. *Env Microbiol Rep* **2**:140–144.

Stauder, M., Huq, A., Pezzati, E., Grim, C.J., Ramoino, P., Pane, L, *et al*. (2012). Role of GbpA protein, an important virulence-related colonization factor, for *Vibrio cholerae*'s survival in the aquatic environment. *Env Microbiol Rep* **4**:439-445.

Stepanovic, S., Vukovic, D., Dakic, I., Savic, B. *and* Svabic-Vlahovic, M. *(*2000*)* A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J Microbiol Meth* **40**, 175–179.

Tall, A., Hervio-Heath, D., Teillon, A., Boisset-Helbert, C., Delesmont, R., Bodilis, J., *et al.* (2013). Diversity of *Vibrio* spp. isolated at ambient environmental temperature in the eastern English channel as determined by *pyrH* sequencing. *J Appl Microbiol* **114 (6)**: 1713-1724.

Thompson, J.R., Randa, M.A., Marcelino, L.A., Tomita-Mitchell, A., Lim, E., and Polz, M.F. (2004) Diversity and dynamics of a north Atlantic coastal *Vibrio* community. *Appl Environ Microbiol* **70**: 4103–4110.

Viaroli, P., Giordani, G., Bartoli, M., Naldi, M., Azzoni, R., Nizzoli, D., *et al*. (2006) The Sacca di Goro Lagoon and an Arm of the Po River. *Hdb Env Chem* **5**:197–232.

Vezzulli. L., and Fabiano, M., (2006) Sediment biochemical and microbial variables for the evaluation of trophic status along the Italian and Albanian Continental Shelves. *J Mar Biol Assoc UK* **86**: 27-37.

902 Vezzulli, L., Guzmán, C.A., Colwell, R.R., and Pruzzo, C. (2008). Dual role
903 colonization factors connecting *Vibrio cholerae*'s lifestyles in human and aquatic
904 environments open new perspectives for combating infectious diseases. Curr Opin
905 Biotech, **19**: 254-259.
906
907 Vezzulli, L., Pezzati, E., Moreno, M., Fabiano, M., Pane, L., and Pruzzo C (2009)
908 Benthic ecology of *Vibrio* spp. and pathogenic *Vibrio* species in a coastal Mediterranean
909 environment (La Spezia Gulf, Italy). *Microb Ecol* **58**:808–818.
910
911 Vezzulli, L., Pruzzo, C., Huq, A., and Colwell, R.R. (2010) Environmental reservoirs of
912 *Vibrio cholerae* and their role in cholera. *Env Microbiol Rep* 2:27–33.
913
914 Xu, H.S., Roberts, N., Singleton, F.L., Attwell, R.W., Grimes, D.J., and Colwell, R.R.
915 (1982) Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in
916 the estuarine and marine environment. *Microb Ecol* **8**: 313– 323.
917
918 Waechter, M., Le Roux, F., Nicolas, J.L., Marissal, E., and Berthe, F. (2002)
919 Characterisation of *Crassostrea gigas* spat pathogenic bacteria. *CR Acad Sci* **325**:231–
920 238.
921
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924
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)**
V. aestuarianus 01/032	control	$6.0x10^{4} \pm 5x10^{3}$	$3.3x10^{2} \pm 4x10^{1}$

	+GlcNac	$2.3x10^{4} \pm 1x10^{3}$ (62% inhibition)	nd
	GlcNac pretreatment	$2.9x10^{4} \pm 4x10^{3}$ (51% inhibition)	nd
	+D-mannose	$2.7x10^{4} \pm 3x10^{3}$ (55% inhibition)	nd
	D-mannose pretreatment	$3.4x10^{4} \pm 5x10^{3}$ (43% inhibition)	nd
V. splendidus LGP32	control	$1.2x10^{5} \pm 2x10^{4}$	$2.4x10^{3} \pm 3x10^{2}$
	+GlcNac	$1.4x10^{5} \pm 1x10^{4}$ (–)	nd
	GlcNac pretreatment	$1.5x10^{5} \pm 2x10^{4}$ (–)	nd
	+D-mannose	$1.6x10^{5} \pm 2x10^{4}$ (–)	nd
	D-mannose pretreatment	$1.5x10^{5} \pm 1x10^{4}$ (–)	nd
V. cholerae O1 14034	control	$9.1x10^{6} \pm 0{,}9x10^{6}$	$5.0x10^{4} \pm 7x10^{3}$

Figure captions

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

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**

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

Supplementary Material

Supplementary Table 1

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

Isolate	Matrix	Date	Species	*mshA*	*gbpA*	*ompU*	*vsm/vam*

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

Supplementary Figure 1

Scatter plots showing relationships existing between *V. splendidus* clade bacteria, *V. aestuarianus,* and *Vibrio* spp. in the different environmental matrices and bivalves and

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

Supplementary Figure 2

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

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

****REVISED****

Luigi Vezzulli[1+], Elisabetta Pezzati[1+], Monica Stauder[1], Laura Stagnaro[1], Paola Venier[2], Carla Pruzzo[1]*

[1]Department of Life, Earth and Environmental Sciences (DISTAV), University of Genoa, Genoa, Italy
[2] Department of Biology, University of Padova, Padova, Italy

+ these authors contributed equally to this work

*Correspondence: Carla Pruzzo, Department of Life, Earth and Environmental Sciences (DISTAV), University of Genoa, Corso Europa, 26, Genoa 16132, Italy. Phone: +39-010-3538251; fax: +39-010-3538267; e-mail: carla.pruzzo@unige.it

Journal: Environmental Microbiology

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

Abstract

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

Introduction

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

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

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

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

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

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

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

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

Results and Discussion

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

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

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

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

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

Survival strategies in the aquatic environment: adhesion to environmental chitin surfaces

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

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

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

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

Survival strategies in the aquatic environment: biofilm formation

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

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

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

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

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

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

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

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

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

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

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

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

and mussels; when present, their concentration ranged from $1.1x10^5$ to $1.5x10^7$ cells/g and from $2.8x10^2$ to $3.8x10^7$ cells/g for oyster and mussel respectively.

In seawater and sediment compartments, when present, their concentration ranged from $1.9x10^1$ to $7.6x10^2$ cells/ml and from $7.4x10^2$ to $1.6x10^5$ cells/g, respectively. The sediment was the compartment where *V. splendidus*-clade bacteria were more frequently found during the study period (9 out of 11 sampling dates) and may thus represents a suitable niche for their persistence in the environment. *V. splendidus*-clade bacteria were also found associated to a variety of other environmental substrates commonly found in the lagoon including large benthic crustaceans (*Dyspanopeus sayi*, *Upogebia pusilla*, *Cratigon* sp., *Carcinus aestuarii*), *Gracilaria verrucosa* macroalgae and *Tapes philippinarum* clam (data not shown).

In contrast to *V. splendidus*, *V. aestuarianus* cells were rarely detected in the Goro lagoon samples. In particular, high concentrations of *V. aestuarianus* bacteria were found associated to zooplankton during warm months, with values ranging from $1.4x10^5$ to $6.9x10^6$ cells/g, and only sporadically found in mussels, oysters, seawater and sediments (**Fig. 8**). In particular, during cold months, *V. aestuarianus* was detected only once (January 2013) in the sediment compartment. The sediment may thus represent an environmental reservoir also for this species where, in accordance with results from laboratory experiments (**Fig. 2**), the bacteria can find a favorable environment for overwintering (Vezzulli *et al*., 2009).

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

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

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

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

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

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

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

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

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

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

Concluding remarks

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

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

Experimental procedures

In vitro studies

Bacterial strains and culture conditions

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

Setting of experimental microcosms for "survival" and "VBNC induction" experiments

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

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

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

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

Nucleic acid extraction

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

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

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

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

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

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

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

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

Enumeration of total and viable bacterial cells by Epifluorescence microscopy

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

Enumeration of culturable bacterial cells

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

Adhesion assays

To assess bacterial attachment to chitin particles one volume bacterial suspension ($5x10^7$ cell/ml) was added to UV-sterilized chitin purified from crab shell (2.5 mg; Sigma Chemical Co., St Louis, MO, USA) and the mixture was incubated for 1 h at 20°C with shaking. A sample without chitin was also prepared as a control. After incubation, chitin particles were repeatedly washed (x3) with 1 ml ASW to remove

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

To assess bacterial attachment to copepods a similar protocol was followed. *T. fulvus* copepods belonging to the *Harpacticoida* family were cultured in filter-sterilized ASW and fed with unicellular phytoplanktonic algae (*Tetraselmis suecica*). Before the association assay, the copepods were vigorously washed 10 times with ASW to remove most adhering bacteria and heat killed (65°C per 15 min). One ml of bacterial suspension ($1x10^8$ cell/ml) was then added to 10 washed copepods in a tube and incubated at room temperature over night. After overnight incubation, copepods were collected and gently washed three times with phosphate buffered saline (PBS) solution (137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) PBS to remove non-adherent bacteria. 200 ul of PBS was then added to the washed copepods and DNA was extracted as previously described. The number of attached bacteria were determined by Real Time PCR. All adhesion assays were performed in triplicate.

Biofilm assays

For biofilm formation analysis, the microtiter plate method (Stepanovic *et al*., 2000) was followed with few modifications. Overnight bacterial cultures grown in ZobellMarine Broth 2216 were diluted to Abs_{640} = 0.02. Sterilized microtitre plates (96 wells) made of PVC were previously conditioned with 200 µl ASW (overnight incubation at room temperature). Each well was filled with 180 µl Zobell medium and 20 µl of the cell suspension and incubated for 24, 48, 72 h and 168 h at 18°C and 24°C. Biofilm was gently washed to eliminate non-adherent bacteria and stained with 0.01% crystal violet (100 µL). After 15 min incubation at room temperature, wells were gently washed with water, and bound dye was extracted from stained cells by adding 200 µl of ethanol : acetone (8:2) per well. After about 1 h incubation, biofilm formation was quantified by measuring the absorbance (Abs) of the solution at 545 nm.

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

In field studies

Experimental design and field sampling

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

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

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

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

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

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

The most-probable-number technique was used to enumerate the number of culturable *Vibrio* spp., in the water, zooplankton and sediment samples. Three dilution series (1:10) were carried out in five replicate culture tubes, starting with 0.5 ml of prepared samples and 4.5 ml of Alkaline Peptone Water (APW, final pH 8.6± 0.2) as the growth medium. Tubes were incubated at 24°C for 8 h in the dark and growth was assessed by plating each APW tube onto Thiosulfate Citrate Bile Sucrose (TCBS) (Conda laboratories, Madrid, Spain) plates. Plates were incubated at 20°C for 48 to 96h. For enumeration of *Vibrio* spp. in oysters and mussels, pools of 30 animals were homogenized in a Polytron PT 3000 Kinematica AG homogenizer and dilutions to the 1/100 and 1/10.000 were performed in ASW. One hundred µl of those dilutions were plated on TCBS plates and incubated for 48 to 96 h at 20°C. Microorganisms were counted as *Vibrio* spp. if they formed 2-3 mm yellow or green colonies on TCBS 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 GAAATTCTACCCCCCTCTACAG, 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-TATTACTCCCGCGCAAAATC 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

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

Environmental variables

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

Statistical tests

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

Acknowledgements

The authors would like to thank Stefano Gualdi (University of Genoa, Italy) for his assistance in the conduction of microcosm experiments, Prof. Luigi Pane (DISTAV, University of Genoa, Italy) for kindly providing *T. fulvus* copepods and Dr. Edoardo Turolla (Istituto Delta, Goro, Italy) for technical help in the establishment of the experimental station in the Goro lagoon and support during field sampling. Authors of this manuscript have no conflict of interest to declare. This work was promoted and funded by the FP7-European project BIVALIFE "Controlling infectious diseases in oysters and mussels in Europe" (Grant Agreement 266157)

References

Balbi, T., Fabbri, R., Cortese, K., Smerilli, A., Ciacci, C., Grande., C. *et al.* (2013) Interactions between Mytilus galloprovincialis hemocytes and the bivalve pathogens *Vibrio aestuarianus* 01/032 and *Vibrio splendidus* LGP32 (2013) Fish Shellfish Immun 35(6):1906 -1915.

Binesse, J., Delsert, C., Saulnier, D., Champomier-Verge`s, M.C., Zagorec, M., Munier-Lehmann, H., *et al* (2008) Metalloprotease *vsm* is the major determinant of toxicity for extracellular products of *Vibrio splendidus*. *Appl Environ Microbiol* **74**:7108–7117.

Chiavelli, D.A., Marsh, J.W., and Taylor, R.K. (2001) The mannose-sensitive hemagglutinin of *Vibrio cholerae* promotes adherence to zooplankton. *Appl Environ Microbiol* **67**:3220-3225.

Duperthuy, M., Binesse, J.D., Le Roux, F., Romestand, B., Caro, A., Got, P., *et al*. (2010) The major outer membrane protein OmpU of *Vibrio splendidus* contributes to host antimicrobial peptide resistance and is required for virulence in the oyster *Crassostrea gigas*. *Environ Microbiol* **12**(4): 951-963.

Duperthuy. M,, Schmitta, P., Garzónd, E., Caro, A., Rosa, R.D., Le Rouxe, F., *et al* (2011) Use of OmpU porins for attachment and invasion of *Crassostrea gigas* immune cells by the oyster pathogen *Vibrio splendidus. P Natl Acad Sci Usa* **108**: 2993-2998.

FAO 2013 Global Aquaculture Production Statistics for the year 2011 (http://www.fao.org/figis/servlet/TabLandArea?tb_ds=Aquaculture&tb_mode=TABLE&tb_act=SELECT&tb_grp=COUNTRY)

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

Gay, M., Renault, T., Pons, A.M., and Le Roux, F. (2004b). Two *Vibrio splendidus* related strains collaborate to kill *Crassostrea gigas*: taxonomy and host alterations. *Dis Aquat Organ* **62**:65–74.

Garnier, M., Labreuche, Y., and Nicolas, J.L. (2008) Molecular and phenotypic characterization of *Vibrio aestuarianus* subsp. *francensis* subsp. nov., a pathogen of the oyster *Crassostrea gigas*. *Syst Appl Microbiol* **31**:358e65.

Hall-Stoodley, L., and Stoodley, P. (2005) Biofilm formation and dispersal and the transmission of human pathogens. *Trends Microbiol* **13**: 7–10.

Hobbie, J.E., Daley, R.J., and Jasper, S., (1977). Use of Nuclepore filters for counting bacteria by fluorescence microscopy. *Appl Environ Microbiol* **33**:1225–1228.

Huq, A., Whitehouse, C.A., Grim, C.J., Alam, M., Colwell, R.R. (2008) Biofilms in water, its role and impact in human disease transmission. *Curr Opin Biotech* **19**: 244-247.

IFREMER report (2013) *Vibrio splendidus* et *V. aestuarianus* detection by Real Time Polymerase Chain Reaction European Union Reference Laboratory for Molluscs Diseases. Edition n°1, Laboratoire de Génétique et Pathologie des Mollusques Marins, Av. de Mus de Loup,
17390 La Tremblade France
http://www.eurlmollusc.eu/content/download/72924/948279/file/Vsplendidus&aestuarianus%20_RealTimePCR.pdf

Johnson, C.N., Flowers, A.R., Noriea III, N.F., Zimmerman, A.M., Bowers, J.C., DePaola, A., *et al*. (2010) Relationships between environmental factors and pathogenic vibrios in the northern gulf of Mexico. *Appl Environ Microbiol* **76**(21): 7076-7084.

Labreuche, Y., Le Roux, F., Henry, J., Zatylny, C., Huvet, A., Lambert, C., *et al* (2010). *Vibrio aestuarianus* zinc metalloprotease causes lethality in the Pacific oyster *Crassostrea gigas* and impairs the host cellular immune defenses. *Fish Shellfish Immun* **29**: 753-758.

Lacoste, A., Jalabert, F., Malham, S., Cueff, A., Gélébart, F., Cordevant, C., Lange, M., and Poulet, S.A. (2001) A *Vibrio splendidus* strain is associated with summer mortality of juvenile oysters *Crassostrea gigas* in the Bay of Morlaix (North Brittany, France). *Dis Aquat Org* 46:139–145.

Le Roux, F., Gay, M., Lambert, C., Waechter, M., Poubalanne, S., Chollet, B., Nicolas, J.L., and Berthe, F.C.J. (2002) Comparative analysis of *Vibrio splendidus*-related strains isolated during *Crassostrea gigas* mortality events. *Aquat Living Resour* **15**:251–258.

Le Roux, F., Binesse, J., Saulnier, D., Mazel, D., (2007) Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene *vsm* by use of a novel counterselectable suicide vector. *Appl Environ Microbiol* **73**:777e84.

Liu, R., Qiu, L., Yu, Z., Zi, J., Yue, F., Wang, L., *et al* (2013) Identification and characterization of pathogenic *Vibrio splendidus* from Yesso scallop (*Patinopecten yessoensis*) cultured in a low temperature environment. *J Invertebr Pathol* **114(2)**:144-150.

Luna, G.M., Dell'Anno, A., and Danovaro, R., (2006) DNA extraction procedure: a critical issue for bacterial diversity assessment in marine sediments. *Environ Microbiol* **8**:308-320.

Matz, C., McDougald, D., Moreno, A.M., Yung, P.Y., Yildiz, F.H., Kjelleberg, S., (2005) Biofilm formation and phenotypic variation enhance predation-driven persistence of *Vibrio cholerae*. *Proc Natl Acad Sci USA* **102(46)**:16819-16824.

Meibom, K.L., Li, X.B., Nielsen, A.T., Wu, C.Y., Roseman, S., and Schoolnik, G.K. (2004) The *Vibrio cholerae* chitin utilization program. *Proc Natl Acad Sci USA* **101**: 2524–2529.

Moreno, M., Vezzulli, L., Marin, V., Laconi, P., Albertelli, G., and Fabiano, M., (2008) The use of meiofauna diversity as indicators of pollution in harbours. *ICES J Mar Sci* **65**: 1428-1435.

Moreno, M., Semprucci, F., Vezzulli, L., Balsamo, M., Fabiano, M., and Albertelli, G., (2011). The use of nematodes in assessing ecological quality status in the Mediterranean coastal ecosystems. *Ecol Indic* **61**: 417-421.

838 Oliver, J.D. (2005). The viable but nonculturable state in bacteria. **43**: 93-100.
839
840 Oliver, J.D. (2010). Recent findings on the viable but nonculturable state in pathogenic
841 bacteria. *FEMS Microbiol Rev* **34**:415-425.
842
843 Oliver, J.D., Pruzzo, C., Vezzulli, L., Kaper, J.B., (2013) *Vibrio* Species," Food
844 Microbiology: Fundamentals and Frontiers, 4th Ed. Edited by M. P. Doyle and R. L.
845 Buchanan 2013 ASM Press, Washington, D.C. doi:10.1128/9781555818463.ch16
846
847 Pernet, F., Barret, J., Le Gall, P., Corporeau, C., Dégremont, L., Lagarde, F., *et al*.
848 (2012) Mass mortalities of Pacific oysters *Crassostrea gigas* reflect infectious diseases
849 and vary with farming practices in the Mediterranean Thau lagoon, France. *Aquacult*
850 *Environ Interact* **2**: 215–237.
851
852 Pruzzo, C., Tarsi, R., Lleò, M.M., Signoretto, C., Zampini, M., Pane, L., *et al.* (2003)
853 Persistence of adhesive properties in *Vibrio cholerae* after long-term exposure to sea
854 water. *Environ Microbiol* **5(10)**:850-858.
855
856 Pruzzo, C., Vezzulli, L., and Colwell, R.R., (2008) Global impact of *Vibrio cholerae*
857 interactions with chitin. *Environ Microbiol* **10(6)**:1400–1410.
858
859 Samain, J.F., and McCombie, H. (2008). Summer mortality of Pacific oyster
860 *Crassostrea gigas*. The Morest Project. Versailles: Quae Editions. 379 pp. SAS Institute
861 Inc. 1995. Basis statistics using SAS/STAT, software course notes. Cary, NC: SAS
862 Institute. 720 pp.
863
864 Saulnier, D., De Decker, S., Haffner, P., Cobret, L., Robert, M., and Garcia, C. (2010)
865 A Large-Scale Epidemiological Study to Identify Bacteria Pathogenic to Pacific Oyster
866 *Crassostrea gigas* and correlation between virulence and metalloprotease-like activity.
867 *Microb Ecol* **59**:787-798.
868

Sechi, L.A., Duprè, I., Deriu, A., Fadda, G., and Zanetti, S. (2000) Distribution of *Vibrio cholerae* virulence genes among different Vibrio species isolated in Sardinia, Italy. *J Appl Microbiol* **88**: 475–481.

Stauder, M., Vezzulli, L., Pezzati, E., Repetto, B., and Pruzzo C (2010) Temperature affects *Vibrio cholerae* O1 El Tor persistence in the aquatic environment via an enhanced expression of GbpA and MSHA adhesins. *Env Microbiol Rep* **2**:140–144.

Stauder, M., Huq, A., Pezzati, E., Grim, C.J., Ramoino, P., Pane, L, *et al*. (2012). Role of GbpA protein, an important virulence-related colonization factor, for *Vibrio cholerae*'s survival in the aquatic environment. *Env Microbiol Rep* **4**:439-445.

Stepanovic, S., Vukovic, D., Dakic, I., Savic, B. *and* Svabic-Vlahovic, M. *(*2000*)* A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J Microbiol Meth* **40***,* 175–179.

Tall, A., Hervio-Heath, D., Teillon, A., Boisset-Helbert, C., Delesmont, R., Bodilis, J., *et al.* (2013). Diversity of *Vibrio* spp. isolated at ambient environmental temperature in the eastern English channel as determined by *pyrH* sequencing. *J Appl Microbiol* **114 (6)**: 1713-1724.

Thompson, J.R., Randa, M.A., Marcelino, L.A., Tomita-Mitchell, A., Lim, E., and Polz, M.F. (2004) Diversity and dynamics of a north Atlantic coastal *Vibrio* community. *Appl Environ Microbiol* **70**: 4103–4110.

Viaroli, P., Giordani, G., Bartoli, M., Naldi, M., Azzoni, R., Nizzoli, D., *et al*. (2006) The Sacca di Goro Lagoon and an Arm of the Po River. *Hdb Env Chem* **5**:197–232.

Vezzulli. L., and Fabiano, M., (2006) Sediment biochemical and microbial variables for the evaluation of trophic status along the Italian and Albanian Continental Shelves. *J Mar Biol Assoc UK* **86**: 27-37.

Vezzulli, L., Guzmán, C.A., Colwell, R.R., and Pruzzo, C. (2008). Dual role colonization factors connecting *Vibrio cholerae*'s lifestyles in human and aquatic environments open new perspectives for combating infectious diseases. Curr Opin Biotech, **19**: 254-259.

Vezzulli, L., Pezzati, E., Moreno, M., Fabiano, M., Pane, L., and Pruzzo C (2009) Benthic ecology of *Vibrio* spp. and pathogenic *Vibrio* species in a coastal Mediterranean environment (La Spezia Gulf, Italy). *Microb Ecol* **58**:808–818.

Vezzulli, L., Pruzzo, C., Huq, A., and Colwell, R.R. (2010) Environmental reservoirs of *Vibrio cholerae* and their role in cholera. *Env Microbiol Rep* 2:27–33.

Xu, H.S., Roberts, N., Singleton, F.L., Attwell, R.W., Grimes, D.J., and Colwell, R.R. (1982) Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. *Microb Ecol* **8**: 313– 323.

Waechter, M., Le Roux, F., Nicolas, J.L., Marissal, E., and Berthe, F. (2002) Characterisation of *Crassostrea gigas* spat pathogenic bacteria. *CR Acad Sci* **325**:231–238.

Table 1

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

Strain	**Experimental conditions**	**Attachment to chitin (bacteria per 2,5 mg of chitin particles)**	**Attachment to copepods (bacteria per copepod)**
V. aestuarianus 01/032	control	$6.0x10^{4} \pm 5x10^{3}$	$3.3x10^{2} \pm 4x10^{1}$
	+GlcNac	$2.3x10^{4} \pm 1x10^{3}$ (62% inhibition)	nd
	GlcNac pretreatment	$2.9x10^{4} \pm 4x10^{3}$ (51% inhibition)	nd
	+D-mannose	$2.7x10^{4} \pm 3x10^{3}$ (55% inhibition)	nd
	D-mannose pretreatment	$3.4x10^{4} \pm 5x10^{3}$ (43% inhibition)	nd
V. splendidus LGP32	control	$1.2x10^{5} \pm 2x10^{4}$	$2.4x10^{3} \pm 3x10^{2}$
	+GlcNac	$1.4x10^{5} \pm 1x10^{4}$ (–)	nd
	GlcNac pretreatment	$1.5x10^{5} \pm 2x10^{4}$ (–)	nd
	+D-mannose	$1.6x10^{5} \pm 2x10^{4}$ (–)	nd
	D-mannose pretreatment	$1.5x10^{5} \pm 1x10^{4}$ (–)	nd
V. cholerae O1 14034	control	$9.1x10^{6} \pm 0,9x10^{6}$	$5.0x10^{4} \pm 7x10^{3}$

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

Figure 6

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

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

Supplementary Material

Supplementary Table 1

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

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

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

Supplementary Figure 1

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

Supplementary Figure 2

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

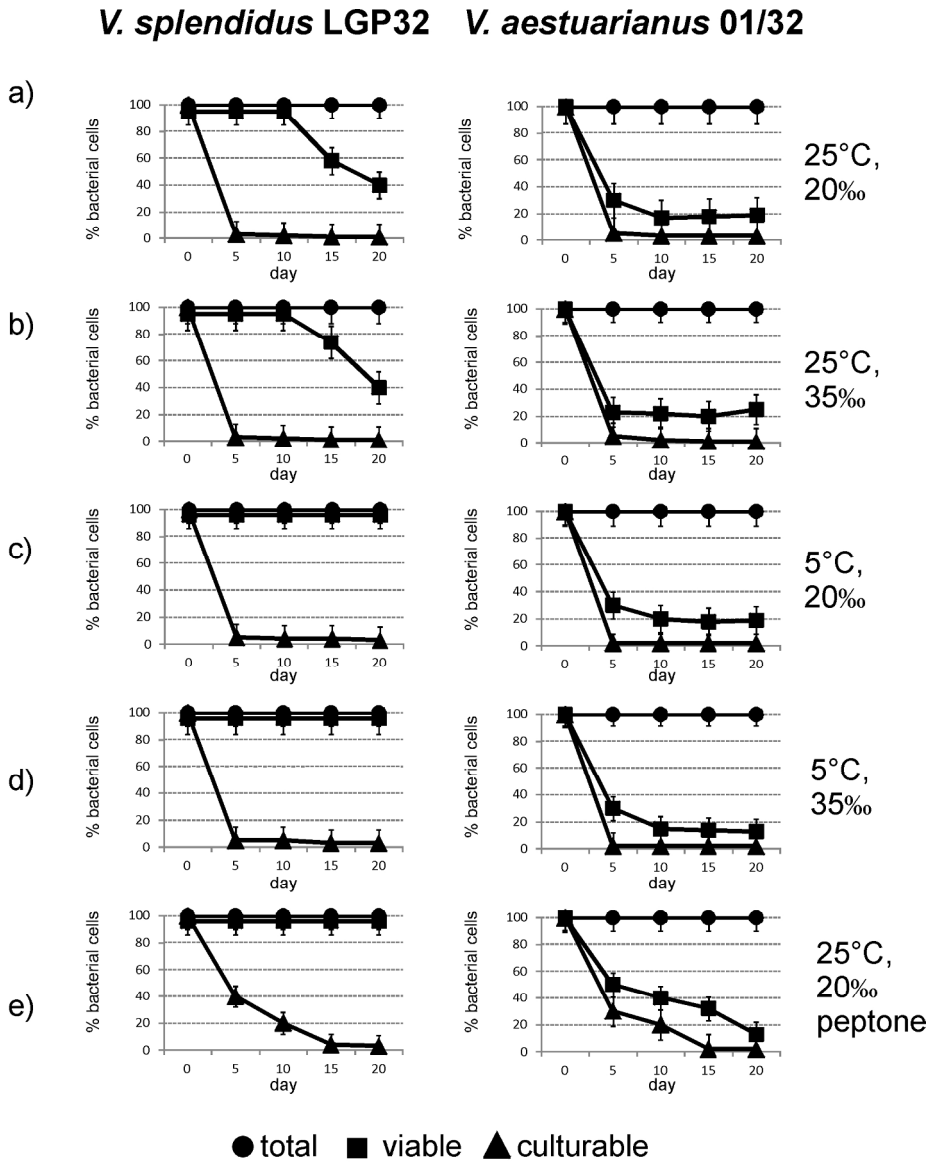

Figure 1
Temporal variation in the percentage of total (black circle), viable (black square) and culturable (black triangle) V. splendidus LGP32 and V. aestuarianus 01/32 cells incubated in seawater under aseptic conditions and at different temperature, salinity and nutrient concentration: 25°C, 20‰ 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

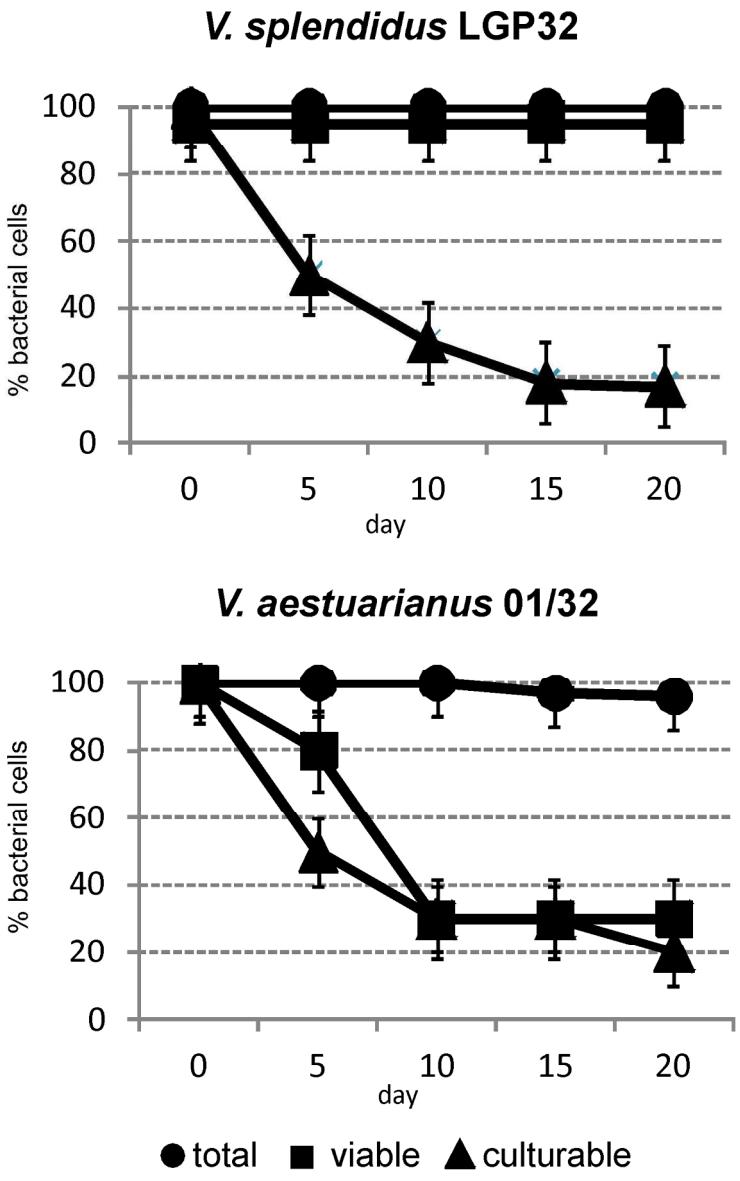

Figure 2
Temporal variation in the percentage of total (black circle), viable (black square) and culturable (black triangle) V. splendidus LGP32 and V. aestuarianus 01/32 cells incubated in the sediment under aseptic conditions at 5°C, 20‰ salinity. Results are the average of three replicate experiments

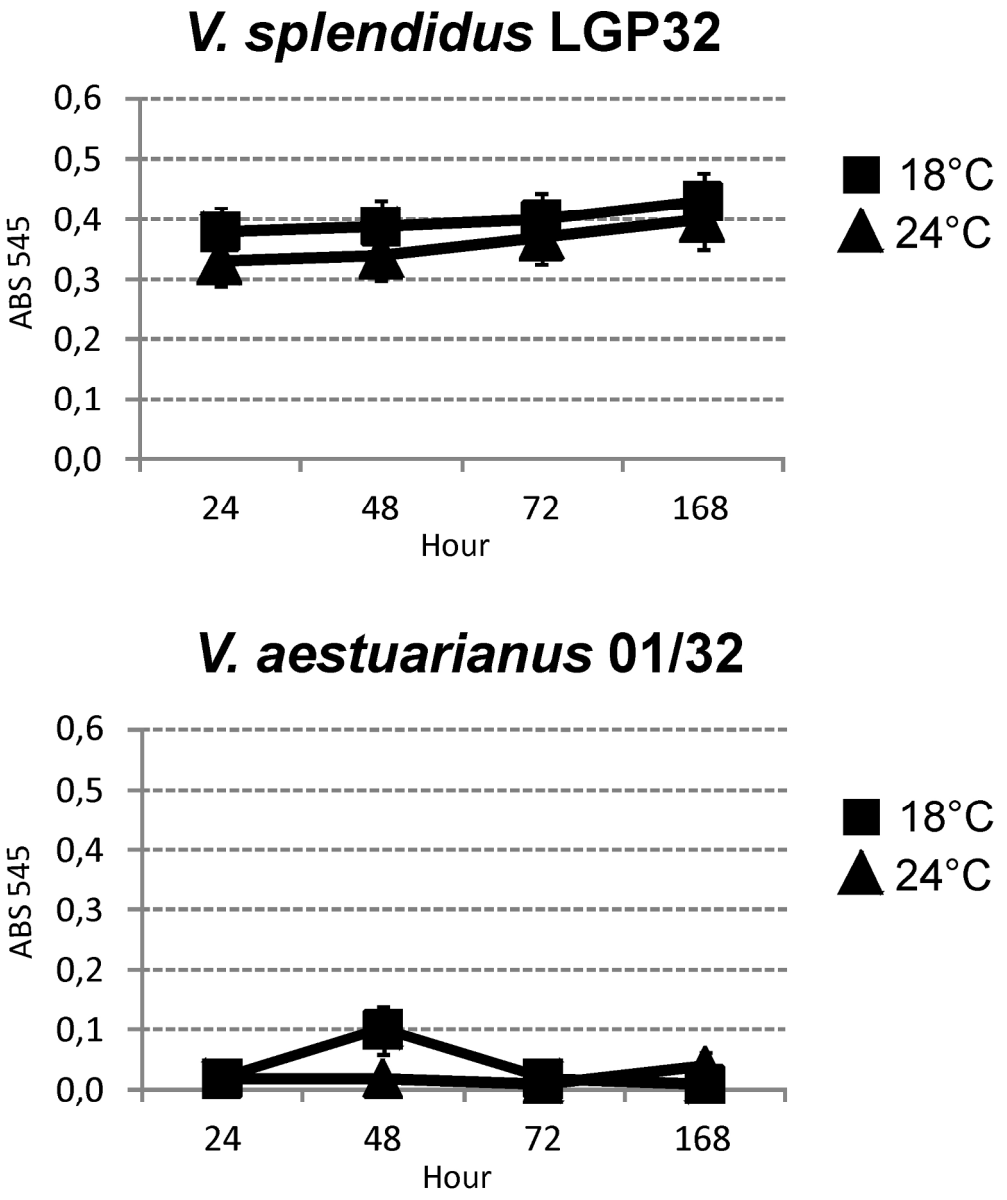

Figure 3
Biofilm formation of V. splendidus LGP32 and V. aestuarianus 01/32 strains on PVC surfaces at 18°C (black square) and 24°C (black triangle). Results are the average of three replicate experiments

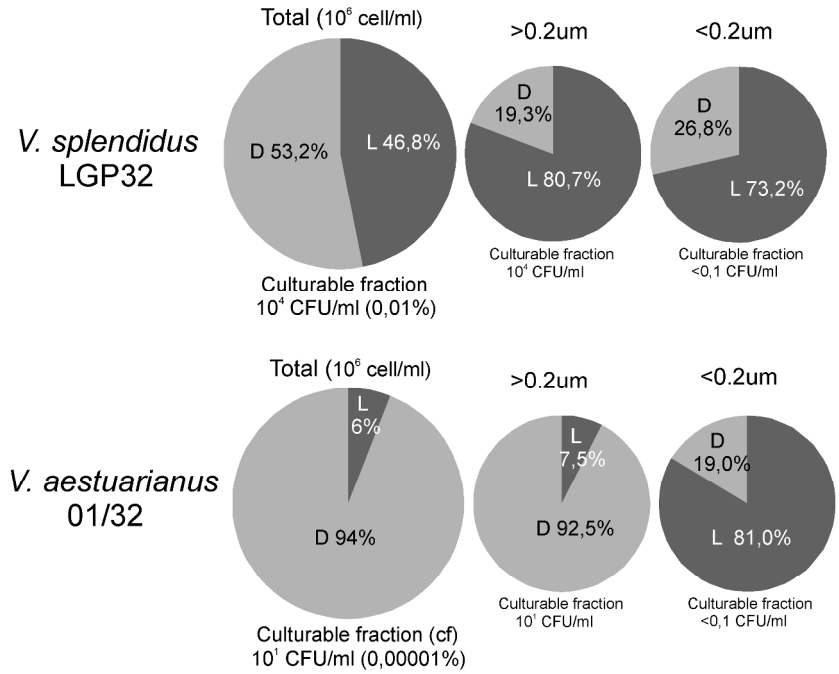

Figure 4
Percentage of live (L) and dead (D) cells of V. splendidus LGP32 and V. aestuarianus 01/32 strains calculated on total, >0.2µm and <0.2µm cell size fractions after prolonged exposure (90 days) to ASW (20‰ salinity) at 5 °C under starving condition. The number of total cells (total) at the beginning of the experiment and the number of culturable cells (culturable fraction) following incubation are also reported

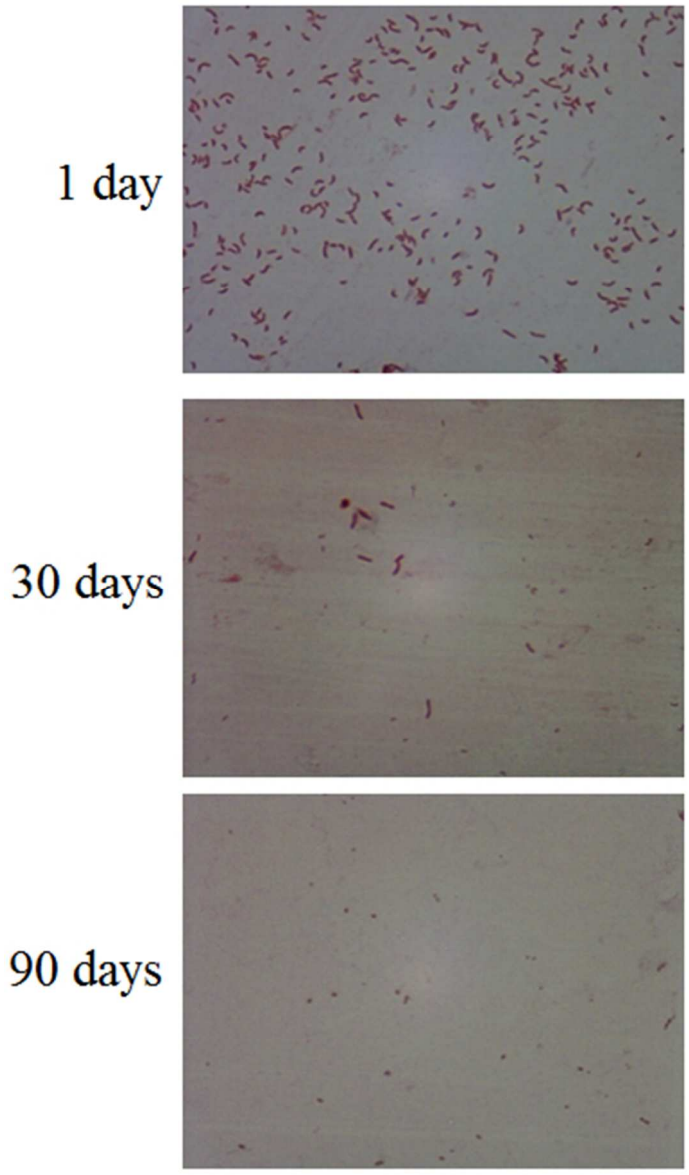

Figure 5
Standard microscopic imaging (magnification 1000X) showing morphological changes observed in V. aestuarianus 01/32 cells following prolonged exposure to ASW (20‰ salinity) at 5 °C under starving condition. Similar results were obtained for V. splendidus LGP32 strains

104x175mm (96 x 96 DPI)

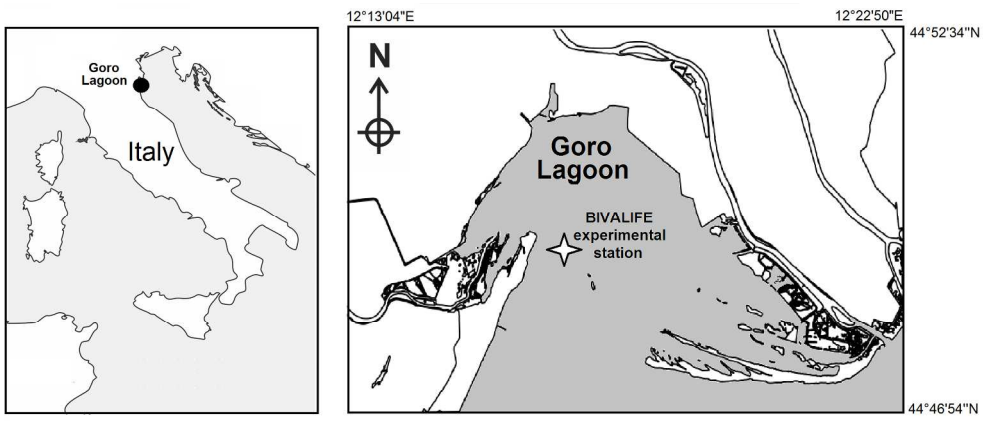

Figure 6
BIVALIFE experimental station in Goro lagoon (Northern Adriatic Sea, Italy)

657x279mm (96 x 96 DPI)

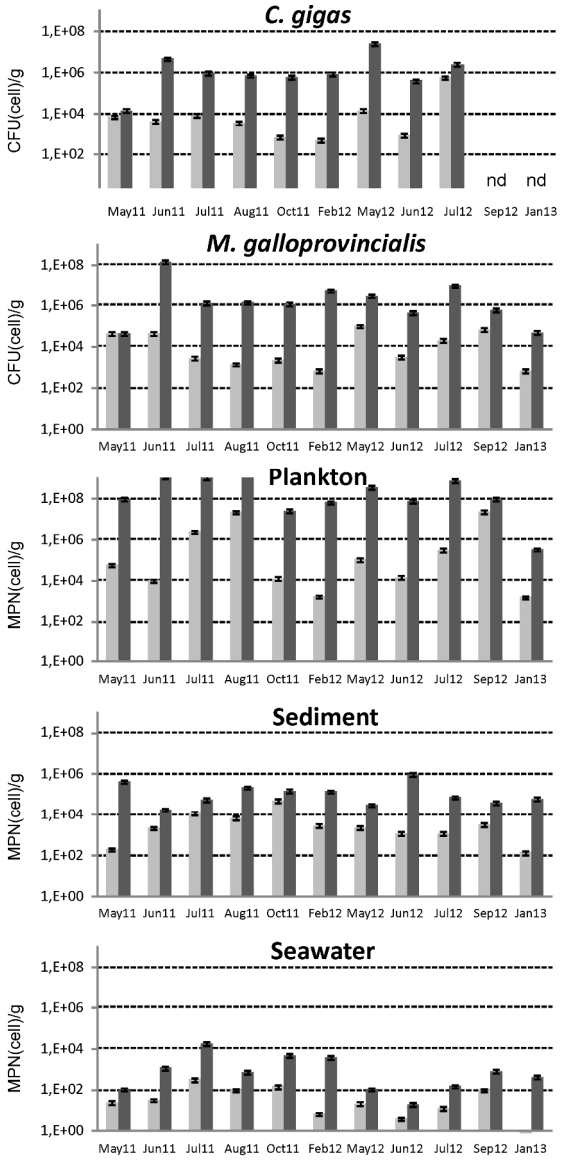

Figure 7
Temporal occurrence of Vibrio spp. enumerated by culture-based (light grey bar) and molecular (dark grey bar) methods in environmental matrices and bivalves collected at the BIVALIFE experimental station in the Goro lagoon

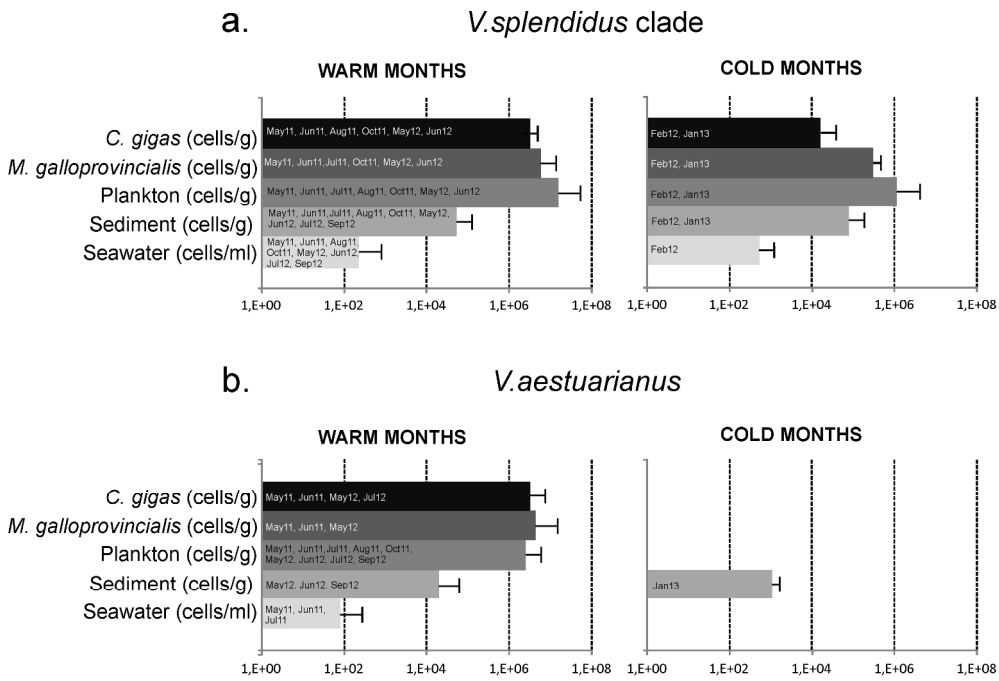

Figure 8
Average Real-Time PCR counts of V. splendidus clade bacteria (a) and V. aestuarianus (c) in warm and cold months in the different environmental matrices and bivalves in the Goro lagoon during the study period (May 2011 to January 2013). Months when the bacteria were detected are superimposed on the histogram bars

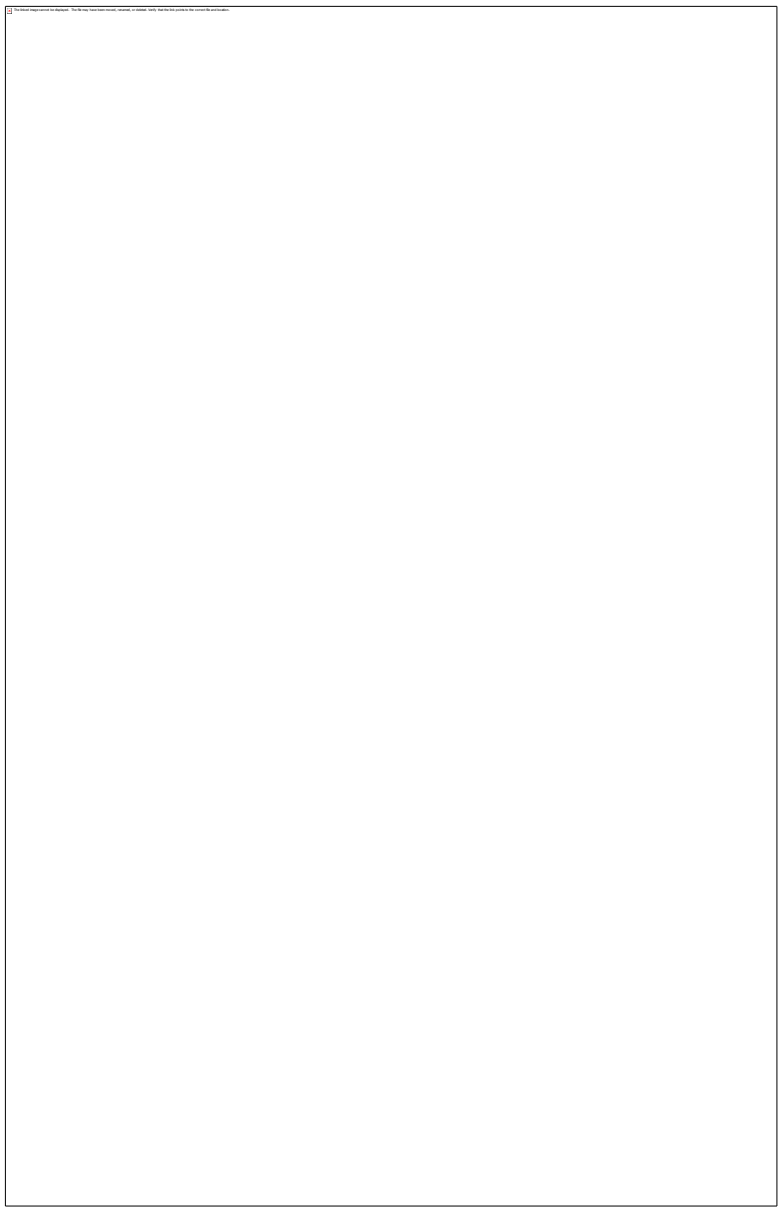

Figure 9
Presence and distribution of genes linked to environmental persistence and virulence in environmental isolates of V. splendidus, V. aestuarianus and other V. splendidus-clade bacterial species isolated from the Goro lagoon