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Running title : A new model to study *V. aestuarianus* pathogenesis

Significance statement

Given the near monoculture of *Crassostrea gigas* in Europe, there is an urgent need to understand the epidemiology of infectious agents that threaten the long-term survival of commercial and natural stocks. Here, exploring the dynamic of oyster infection by *Vibrio aestuarianus* in the wild, we showed that this animal constitutes a reservoir for the bacteria in the winter, at a load that is undetectable by available diagnostic methods. Oysters asymptomatically carrying putative pathogens constitute a risk factor for disease spread when transferred between production areas for farming purposes. We also developed an experimental "ecologically realistic" infection model in a mesocosm, allowing infection by natural route. We demonstrated the importance of haemolymph for initial colonisation and the septicaemic nature of this disease caused by *V. aestuarianus*.

Abstract

Although vibrios are frequently associated with marine organisms mortality outbreaks, knowledge on their ecology and pathogenicity is sparse, thus limiting disease management and prophylactic strategies. Here, we investigated *V. aestuarianus* infection onset and progression in the wild, taking advantage of a "claire" pond: a semi-closed system with limited seawater renewal, theoretically more adapted to disease transmission. We showed a positive association of the bacteria with oysters, which can constitute a reservoir for the bacteria in the winter. Moreover, passage through oysters was found to be necessary for experimental disease reproduction as vibrios shedding from diseased oysters has higher infectivity than from *in vitro* grown. We next developed an experimental "ecologically realistic" infection model in a mesocosm, allowing infection by natural route. By means of this non-invasive protocol, we analysed the pathogenesis of the bacteria and demonstrated the importance of haemolymph for initial colonisation and the septicaemic nature of this disease.

INTRODUCTION

Owing to its potential for rapid growth and its remarkable tolerance to various environmental conditions, the oyster *Crassostrea gigas* has become one of the most important aquaculture animals and is established in many regions of the world (Chaney and Gracey, 2011) representing an important socioeconomic and cultural activity in coastal areas. Nonetheless, the intensification of *C. gigas* farming has coincided with mass mortality events threatening the sustainability of this industry. In France, since 2008, mortality outbreaks of juvenile oysters have considerably increased in frequency and intensity, with mortality levels currently ranging between 60% and 90% (Barbosa Solomieu et al., 2015). Various research efforts have mainly focused on the hypothesis that these events result from the emergence of a new genotype of the herpes virus, OsHV-1 µvar (Segarra et al., 2010; Martenot et al., 2011; Schikorski et al., 2011a; Schikorski et al., 2011b; Martenot et al., 2013; Barbosa Solomieu et al., 2015). On the other hand, it has been observed that rapid colonisation by diverse vibrios precedes replication of OsHV-1 when oysters are incubated in the field during a mortality outbreak (Petton et al., 2015). In the absence of bacteria, a high load of the herpes virus is not sufficient to induce the full manifestation of the disease (Petton et al., 2015), while antibiotic treatment has strongly reduced oyster mortality rates, revealing a role of bacteria in the disease transmission and development (Lemire et al., 2014; Petton et al., 2015; Bruto et al., 2017). Thus, a consensus tends to define oyster juvenile syndrome as a polymicrobial disease involving OsHV-1 µvar and diverse populations of *Vibrio* although the role of each pathogenic agent remains to be determined.

Even more worrying is the increase in frequency of oyster mortality outbreaks associated with the presence of *V. aestuarianus* since 2012 because this vibriosis affects the adult stage of the animal and causes ineluctable losses for the farmers (Travers et al., 2015). Genotyping analyses showed that *V. aestuarianus* strains isolated from diseased oysters cluster into two

nearly identical clonal lineages (A and B), whereas isolates sampled from healthy oysters, cockles, or zooplankton were found to be more diverse (Goudenege et al., 2015). Within each of the two lineages A and B, a majority of strains are virulent, suggesting that a few strains have recently lost their pathogenicity. In line with this hypothesis, a single-nucleotide deletion, inducing a frame shift in the *varS* gene that codes for the transduction histidine-protein kinase of sensory system VarS/VarA (Lenz et al., 2005), was linked to a loss of virulence in one strain. The *varS* gene is intact in all the virulent strains, and genetic analyses confirmed its role in the infection of oysters and in expression of a secreted metalloprotease (Goudenege et al., 2015).

To date, knowledge about the pathogenesis of *V. aestuarianus* infection and especially the route of infection remains scarce (Vezzulli et al., 2015; Le Roux et al., 2016) and has been mainly based on experimental injection involving a single cultivated strain (Labreuche et al., 2006b; Garnier et al., 2007; Labreuche et al., 2010). A gene encoding a metalloprotease (*vam*) has been previously implicated in *V. aestuarianus* virulence as an effector. When expressed from a plasmid by a non-pathogenic *Vibrio* strain (Labreuche et al., 2010), this gene induces the same immunosuppressive effects on haemocytes as those observed for *V. aestuarianus* extracellular products, i.e. inhibits their phagocytic abilities and adhesion capacity (Labreuche et al., 2006a).

However, major questions regarding the ecology and biology of *V. aestuarianus* must be addressed prior to diagnose, predict or eventually prevent oyster disease outbreaks. First, what are the persistence and dynamics of *V. aestuarianus* in the environment? Previous *V. aestuarianus* samplings has been biased toward moribund animals, *i.e.* without taking into consideration the microbial community in the water column to which oysters are connected. *Vibrio* are ubiquitous in marine ecosystems and can adopt different lifestyles from free living to particle-attached or animal-associated (Hunt et al., 2008). A previous study has proposed

that during the cold seasons, *V. aestuarianus* may subsist on the sediment, from which it can emerge in the warmest months (Azandegbe et al., 2010). Second, what is the infection route of *V. aestuarianus*? Intramuscular injection of bacterial suspensions or putative exotoxins into the animal adductor muscle has been widely used in oysters as an experimental infection model, allowing for high-throughput screening of bacterial isolates with good reproducibility. Nevertheless, this technique does not reflect the natural route of infection, thus precluding other factors that may influence the onset and pathology of disease (e.g. chemotaxis, colonisation), and the method of infection by immersion frequently does not result in any mortality at all (Le Roux et al., 2016).

Here, we investigated the disease ecology of *V. aestuarianus*. Oysters were deployed in a pond, a semi-closed system with limited seawater renewal, to characterise the spatiotemporal dynamics of oyster infection by *V. aestuarianus* at fine scale. At different time points, *V. aestuarianus* was detected in distinct habitats (seawater fractions, sediment, and oysters) that are connected by dispersal. Our analysis revealed that *V. aestuarianus* is preferentially associated with oysters, and its presence in healthy oysters can be revealed in cold months by thermal stress. All isolated strains belong to lineage A and are virulent. We next developed an experimental infection system in a mesocosm that enables oyster infection via a natural route, and we described the histopathological damage associated with *V. aestuarianus.*

RESULTS AND DISCUSSION

Natural infection by *V. aestuarianus*: an experimental design

To investigate the dynamics of oyster infection in the field, we used specific pathogen free (SPF) oysters that are descendants of a pool of genitors that are produced in hatcheries under

highly controlled conditions (Lemire et al., 2014; Petton et al., 2015; Le Roux et al., 2016). These animals are not axenic, as is the case with gnotobiotic animals (Marques et al., 2006); however, OsHV-1 and *V. aestuarianus* DNAs were not detected, and the load of total vibrios remained low for the SPF oysters analysed before the experiments (<1 colony-forming units [cfu] per milligram of tissue). In October 2014, batches of SPF oysters (herein termed recipients, n = 1500) were placed in two distinct ponds, with the first pond (designated as pond A) containing *V. aestuarianus*-infected oysters (donors, n = 2000), and with the second pond (designated as pond B) containing only SPF oysters (recipients, n = 1500). For 15 weeks, mortality rates were recorded weekly and were observed only in the first pond (donors and recipients). On five dates (weeks 0, 3, 6, 9, 15), 50 living recipients were sampled. Thirty of these animals were used to collect haemolymph, detect the presence of *V. aestuarianus* or OsHV-1 by quantitative PCR (qPCR), and to isolate the bacteria; meanwhile, the 20 remaining oysters were used to allow development of the disease in tanks at 25°C. On five dates, seawater was sampled, and particles were fractionated by size to collect zooplankton (60 µm plankton net), large phytoplankton, and organic particles, smaller organic particles and free-living bacterial cells (5, 1, and 0.22 µm pore size filters, respectively). Sediment and interstitial seawater were also sampled. It should be noticed that *V. aestuarianus* cannot be isolated on *Vibrio*-selective media such as TCBS (Azandegbe et al., 2010). Hence, half of all samples and filters served for DNA extraction and *V. aestuarianus* detection by qPCR and half for bacterial isolation on Zobell agar plates and for subsequent screening of *V. aestuarianus* isolates (see the Materials and Methods section for details).

Spatiotemporal dynamics of oyster infection by *V. aestuarianus*

In pond A, mortality was lower than 10% during the first 3 weeks (Figure 1) and dramatically increased between weeks 3 and 8 thus reaching 42%. Subsequently, mortality slightly

increased during the 8 next weeks, reaching 49% at the end of the experiment. The peak of mortality coincided with a temperature ranging from 15°C to 20°C and salinity ~32 g/L, although correlation between daily mortalities and temperature was weak (R^2=0.49) (Figure 1). In the laboratory, animals collected after staying for 3, 6, 9, and 15 weeks in pond A, experienced cumulative mortality of 60%, 60%, 50%, and 20%, respectively, within the following 7 days in the laboratory (Figure 1, red bar). OsHV-1 DNA was never detected in these animals (data not shown). No mortality was observed among unexposed animals maintained at 25°C in the laboratory.

Total-vibrio and *V. aestuarianus* DNAs were detected in seawater fractions, sediment, and living oyster haemolymph at different time points, i.e. 0, 3, 6, 9, and 15 weeks (Figures 2 and S1). *V. aestuarianus* was never detected in zooplankton. On day 0, *V. aestuarianus* was detected only as free living (10^3 bacteria per litre). After 3, 6, and 9 weeks, the total vibrio load was estimated to be 10^1, 10^2, and 10^3 bacterial cells per litre, respectively, for large phytoplankton and organic particles, smaller organic particles, and free-living bacterial cells. *V. aestuarianus* accounted for up to 1% of all the vibrio cells (*i.e.* genome copies) in some fractions and on some dates of sampling, *i.e.* free living at weeks 0, 3, 6 and 9, smaller organic particles at week 3, 6 and 9, and large phytoplankton and organic particles at week 9 (Table S1). Considering the overall results in seawater, *V. aestuarianus* was found to be differentially distributed in different fractions (p value = 3.22e-05, Kruskal–Wallis test), with preferential association with the planktonic fraction (p values <0.05, pairwise comparisons, Wilcoxon test). This finding suggests that *V. aestuarianus* does not have to be attached to particles or integrated into marine aggregates to infect oysters, as previously demonstrated for *V. vulnificus* (Froelich et al., 2013). In the sediment, *V. aestuarianus* DNA accounted for up to 18% of vibrio cells (5 bacterial cells/mg) but was detected only after 9 weeks (Table S1),

suggesting that the persistence of *V. aestuarianus* in the sediment during the cold season as proposed by Azandegbe et al. (2010) is unlikely.

V. aestuarianus was found to be abundant in oyster haemolymph of living oysters (10^5 bacteria per millilitre of haemolymph) at week 3, 6, and 9, when *V. aestuarianus* was estimated to represent 25 to 30% of all vibrio cells. Fisher's exact test and odds ratio analysis revealed a significant positive correlation between oysters and *V. aestuarianus* ($p = 6.10^{-12}$, odds ratio 95% confidence interval [12.4554; ∞]) suggesting that *V. aestuarianus* is preferentially associated with oyster haemolymph. During the epidemic phase (week 3, 6 and 9), *V. aestuarianus* abundance reached 2.5 to 5.6 x 10^5 bacteria/ml in live oysters and its prevalence was estimated around 50 to 60% (Figures 1 and S1). Of note, at week 15, it was not possible to detect *V. aestuarianus* in oysters sampled in the pond, although 20% of the animals experienced further mortality when maintained in tanks at 25°C (Figures 1 and S1). As *V. aestuarianus* strains were isolated from moribund animals collected in tanks (see below), it appeared that at a low temperature (~5°C, see Figure 1) oysters were infected by *V. aestuarianus* at a load that is undetectable by our diagnostic method (threshold of $<10^2$ bacteria per millilitre) but can be revealed in the laboratory by a thermal stress assay. Such persistence of vibrio at a low concentration in the host has been previously described for the human pathogen *V. parahaemolyticus* (Olafsen et al., 1993; DePaola et al., 2003) and may constitute a risk factor for disease spread when oysters are moved between production areas for farming purposes. Our results are in agreement with other data (Petton et al., 2015), showing that oysters can asymptomatically carry putative pathogens, thus raising concerns about the role of oysters as pathogen reservoirs. We can speculate here that thermal stress in the laboratory can be applied to diagnose asymptomatic carriage of a pathogen (Petton et al., 2015).

We next attempted to isolate *V. aestuarianus* strains from DNA-positive samples. Among 592 colonies isolated from seawater and sediment fractions, only one strain, originating from a free-living fraction and sampled at week 0, was identified as *V. aestuarianus* (Table S2). A total of 212 out of 734 bacteria isolated from oysters collected at weeks 3, 6, and 9 were identified as *V. aestuarianus*, confirming the findings about the abundance and prevalence of this species in the haemolymph of diseased animals. In addition, we were able to isolate 48 *V. aestuarianus* strains from moribund animals sampled in the tank after the development of the disease by thermal stress. All the 261 isolated *V. aestuarianus* strains belong to clade A (Goudenege et al., 2015) with 100% identity within the partial sequences of *ldh* previously used to differentiate the two virulent lineages within the species. A total of 227 strains (including the strain isolated in seawater) were tested for virulence by injection of 10^2 bacteria into oysters, and all of them induced high mortality, i.e. > 70% (data not shown). Genotyping and virulence assays did not reveal a diversity of the strains infecting oysters and may suggest a clonal expansion of infected animal by virulent genotype(s)

Infection by *V. aestuarianus* in a mesocosm

We previously observed that *V. aestuarianus* is transmitted from donor to recipient oysters mainly as a free-living bacterium. We next determined whether an oyster transiently immersed into bacteria-contaminated water would contract the disease. When a virulent *V. aestuarianus* strain (12_016a) cultivated in a rich medium was employed for infection via bath (5×10^8 cfu/L) the obtained results lacked reproducibility, i.e. mortality was observed in six out of 14 distinct experiments (Figure S2). We then hypothesised that virulence of *V. aestuarianus* (and consequently reproducibility of the immersion model of infection) may be improved when bacteria are shed from infected oysters. Oysters were injected with the

12_016a strain (10^7 cfu/animal) and maintained for 48 h under static conditions. *V. aestuarianus* load in contaminated seawater (CSW) was estimated by qPCR and adjusted to 5 × 10^8 cfu/L (for details see Materials and Methods). SPF oysters were then exposed to CSW for 24 h, washed, and transferred to a new tank containing UV-treated water. After this procedure, deaths were observed in 16 out of 17 distinct experiments (Figure S2), suggesting that the virulence of *V. aestuarianus* strengthens *in vivo*. This result has been previously demonstrated on *V. cholerae* in which specific genes are induced *in vivo* and favour further infection by increasing persistence in the stool and/or aquatic environments (Schild et al., 2007). Indeed, the transcriptome of human-shed vibrios differs substantially from that of *in vitro*–grown *V. cholerae*, resulting in a hyper-infectious state (Merrell et al., 2002; Alam et al., 2005; Butler et al., 2006).

V. aestuarianus strain 12_016a and its Δ*varS* derivative (hereafter abbreviated as WT and Δ*varS*, respectively, for simplicity) have been previously shown to induce similar mortality (80–100%) when injected into oysters at a high dose (10^7 cfu/animal), whereas only the wild-type strain induces significant mortality when injected at a low dose (10^2 cfu/animal) (Goudenege et al., 2015). When injected at a high dose, shedding of 1 × 10^8 to 2 × 10^8 bacterial cells/animal was noticed for both strains, resulting in a similar load of WT and Δ*varS* in CSW (thereafter termed CSW_{wt} or CSW_{Δ}) and can thus be used for experimental infection by immersion (Figure 3). By this procedure, when oysters were immersed in CSW_{wt}, mortality was observed after an initial latent phase of 3 days (phase 1), reached 30% on day 6 (phase 2), stabilised for 4 days (phase 3), increased to 40% on day 14 (phase 4), and then ceased. These dynamics suggest that 3–4 days are necessary for freshly shed bacteria to kill an oyster. Immersion in CSW_{Δ} induced maximal mortality of 7% after 7 days, while no deaths occurred when oysters were immersed in UV-treated seawater as a control (Figure 3).

Altogether these results suggest that deletion of the gene encoding regulator VarS affects the ability of *V. aestuarianus* to colonise oysters. In the future, transcriptomic analyses should allow to decipher which genes and biological processes are controlled by VarS and involved in oyster infection.

Dynamics of *V. aestuarianus* infection in the host

Our experimental infection system in a mesocosm enables oyster infection via a non-invasive approach and hence should be of prime interest for identification of the natural route of infection with *V. aestuarianus*.

The kinetics of infection of each strain (WT and Δ*varS*) were first explored by qPCR analysis using DNA extracted from living and moribund animals' tissues and haemolymph (up to 15 animals/strain per sampling point; Figure 4). *V. aestuarianus* was never detected in animals incubated in UV-treated seawater. On day 1, *V. aestuarianus* was detected exclusively in the haemolymph of 73% and 23% of living animals of strains WT and Δ*varS*, respectively. On day 4, *V. aestuarianus* was detected only in the tissues (haemolymph, gills, digestive gland, mantle, muscle, and palps) of living animals infected by the WT strain, whereas this bacterial species could not be detected in the tissues of oysters infected with the Δ*varS* mutant. Here we observed that a Δ*varS* mutant is unable to persist in the hemolymph. In moribund animals infected with WT, we observed an increase of bacterial load by four orders of magnitude in the haemolymph, by comparison with living animals (3×10^4 bacterial cells/haemocyte in moribund oysters vs 10^1 bacterial cells/haemocyte on day 1). In the other tissues, the loads were found to be similar (mean of 5×10^2 bacterial cells in moribund oysters, standard error (SE): 1×10^2). Our results revealed early colonisation of haemolymph by *V. aestuarianus* and its capacity for proliferation in this fluid, thereby causing bacterial spread inside the animals. As previously shown for *V. crassostreae* (Bruto et al., 2017), *V. aestuarianus* is abundant in

haemolymph, considered the most hostile environment in the animals, i.e. containing the immunocompetent cells and various immune effectors and soluble factors produced by the haemocytes to protect the oyster from infections (Schmitt et al., 2010). This situation raises the question of haemolymph colonisation as a common trait of oyster pathogens, although the mechanisms of interaction with haemocytes may be different. For instance, *V. tasmaniensis* strain LGP32 has evolved the ability to survive in haemocytes (Duperthuy et al., 2011) and induces cytolysis by expressing antioxidant and copper resistance mechanisms (Vanhove et al., 2016). *V. aestuarianus,* by contrast, induces haemocyte impairments probably through an oxidative disequilibrium. Moreover, it can adhere to haemocytes (Pezzati et al., 2015), and inhibits cell adhesion and phagocytosis through its extracellular products (Labreuche et al., 2006a), in particular, metalloprotease Vam (Labreuche et al., 2010), thus indicating a strategy of extracellular multiplication (Labreuche et al., 2006b).

V. aestuarianus–associated damage

Histopathological damage and localisation of *V. aestuarianus* in diverse tissues were next confirmed on sacrificed animals, by histology and immunohistochemistry with a polyclonal antibody. Here again, *V. aestuarianus* was never detected in control animals (Figure 5A) presenting normal tissue structures (Figure 6A, C, E).

It is important to note that except in advanced cases of haemocyte infiltration, blood spaces generally appeared empty (Martoja and Martoja, 1967), limiting our interpretation of circulating bacteria at the initial stages of the disease, because of such histological methods.

On day 1, the WT (Figure 5B and Table S3) and Δ*varS* (Table S3) strains were detected as few foci in the gills of 5 out of 14 and 6 out of 10 animals, respectively. Nevertheless, as early as this first day after bacterial contact, first signs of histological damage were observed (Figure 6, Table S4). These lesions could be classified into three classes: (type 1) lysis of the

sub-epithelial connective tissue in the mantle (Figure 6B), (type 2) epithelial atrophy of digestive diverticula (DD) with dilation of the diverticulum lumen (Figure 6D), and (type 3) haemocyte lysis and/or infiltration by haemocytes and/or haemocyte agglutination in haemolymph vessels (Figure 6F–H). Even though each of these lesions cannot be defined as specific to this disease (Couch, 1984; Knowles et al., 2014; Martenot et al., 2016), the presence of all of them in field oysters is generally noticed in *V. aestuarianus*–infected animals sampled during surveillance programs (REPAMO Network, Céline Garcia, personal communication) and should constitute a part of a diagnostic method.

On day 4, Δ*varS* was detected in gills (3 of 10 animals) and rarely in the digestive gland (1 of 10 animals), whereas the bacteria were not detected in the other seven animals, thereby indicating bacterial clearance and/or the absence of initial infection (Table S3). Even though few lesions were detected on day 1 in the animals immersed in the $CSW_{\Delta varS}$, we did not notice any progression of the damage on day 4 either in the percentage of affected animals or in lesion intensity (Table S4). Taken together, these results revealed the absence of progression of the disease in the Δ*varS*-infected animals.

By contrast, in oysters immersed in CSW_{wt} and sampled on day 4, bacteria were detected in most of the individuals (13 of 15). In those samples, two bacterial distribution patterns corresponding to two different disease stages were observed. We thus defined two groups. For 6 of 8 animals (termed Group a, Figure 5C), *V. aestuarianus* was localised to only one tissue: the gills. In contrast, for 7 out of 7 animals (termed Group b, Figure 5D), intense colonisation of all oyster tissues was observed. To be precise, stained bacteria-like cells were detected i) in gills, as attached to epithelial cells or as isolated cells, ii) around the digestive gland in connective tissues as well as attached or inside epithelial cells of the digestive tissue, iii) in

the mantle, in clusters in connective tissues, iv) in between muscular fibres of the adductor muscle, and v) in the periphery of the sinus, vein, or artery of different tissues. The disease progression on day 4 was associated with an increase of affected animals for the 3 types of lesions (Table S4), as well as an increase in lesion intensity (an increase in the number of affected areas and significant atrophy of the DD epithelium as compared to the control). Finally, damage intensity increased reaching a maximum in moribund animals, in which *V. aestuarianus* was detected in every tissue of all the tested animals (Figure 5E). These results confirmed the septicaemic characteristics of *V. aestuarianus* infection.

Finally, the *V. aestuarianus* route of infection can be summarised by 5 steps: (1) early colonisation of haemolymph by a virulent strain, (2) bacterial multiplication in this fluid (3) accompanied by haemocyte recruitment and lysis, and finally (4) invasion of other connective tissues (5) in which damage was noticed. Considering the limited ability of non-virulent strain Δ*varS* to colonise the haemolymphatic system and to invade other tissues, our results point again to the role of haemolymph in pathogen selection. This fluid has, thus, to be considered with interest for future studies dealing with early diagnosis and prophylactic strategies.

MATERIALS AND METHODS

Animals

SPF juveniles were generated as previously described (Petton et al., 2015). A batch of SPF oysters (intended for a field survey) was produced in January and April 2014 (Ifremer Argenton, Brittany, France) and transferred to Ifremer La Tremblade (Charente Maritime, France) in May 2014. At the time of their transfer into ponds (see below) their mean total weight was 19.6 ± 3.8 g (mean ± SD) and their mean size 56.1 ± 4.3 mm. Biparental families of SPF oysters were also produced in January 2015 (Ifremer La Tremblade) for experimental pathological analysis. At the start of the experiment, oysters were 18 months old, weighed 24.2 ± 5.7 g and had a size of 61.2 ± 8.1 mm (mean ± SD). Oysters were maintained in 240 L raceways with a continuous UV-treated seawater flow and an *ad libitum* phytoplankton diet (*Isochrysis galbana*, *Tetraselmis suecica*, and *Skeletonema costatum*).

Twelve oysters were screened before experiments for the eventual presence of *V. aestuarianus* and OsHV-1 herpes virus by standard protocols (Pepin et al., 2008; Saulnier et al., 2009).

Bacteria

Bacteria: strains, growth conditions, and suspensions

V. aestuarianus strain 12/016 (a highly pathogenic strain isolated during an oyster mortality episode) and its mutant variant 12/016 Δ*varS* (a mutation in the *varS* gene) (Goudenege et al., 2015) were used for bacterial challenges in this study. *V. aestuarianus* strains were grown in

Zobell agar (peptone 4 g/L, yeast extract 1 g/L, Tris-HCl buffer 0.5 g/L, ferric citrate 0.1 g/L, agar 15 g/L in 1× artificial seawater, pH 7.4) at 22°C. The *Vibrio* suspension was obtained from an isolate stored at −80°C in the Zobell medium containing 15% glycerol (v/v). The bacterial strain was placed in the liquid Zobell medium and incubated for 24 h at 22°C with constant shaking at 20 rpm. The resulting suspension was centrifuged at 3000 rpm for 10 min at room temperature. The supernatant was discarded, and the pellet was resuspended in sterile artificial sea water (SASW). The bacterial concentration was evaluated spectrophotometrically at 600 nm and adjusted to optical density (OD) of 1.0 (5.10^8 bacterial cells/mL). Purity and concentration of the bacteria were checked by plating on Zobell agar.

Field survey

Natural infection by *V. aestuarianus* in a pond

Next, 1500 SPF juveniles (mean total weight 19.6 ± 3.8 g [mean ± SD]; size 56.1 ± 4.3 mm) were transferred into two non-submersible individual ponds commonly called “claires” in France (La Tremblade, Charente Maritime, France) from October 7th, 2014, to February 1st, 2015. Ponds had an average depth of approximately 70 cm for a volume of 250 m3. The two ponds were 5 km away and were naturally supplemented with seawater during the spring tides, when the tidal coefficient exceeds 85. One of these ponds initially contained 2000 animals supposed to be partially infected with *V. aestuarianus*. Subsequent analyses of those animals confirmed that they were carriers of the bacteria (Lionel Degrémont, personal communication). Mortality and environmental parameters (temperature and salinity) were recorded each week (340i, WTW). At five sampling points (see below), 30 live oysters were collected, and haemolymph was withdrawn for bacterial culture and DNA extraction (see above). Additionally, each week, 20 oysters were sampled and then transferred to the

laboratory located 200 m away from the pond. The oysters were placed in one tank filled with UV-treated seawater, which was aerated and maintained at 25°C. Mortality was recorded daily for 7 days, and moribund animals were removed and analysed by detection and quantification of *V. aestuarianus* and herpes virus OsHV-1. In the second pond, one sampling was performed at week 0. Additionally, animals were submitted to thermal choc on weeks 0, 3 and 6. In absence of V. aestuarianus detection and of any noticeable mortality (in the pond B during the 15 weeks of survey and in lab), additional samplings were not performed.

Seawater and sediment sampling in the pond

Seawater and sediment from the pond were sampled on September 29th, October 27th, November 17th, and December 8th, 2014, as well as on February 1st, 2015. Two litres of seawater was successively filtered at 60, 5, 1, and 0.22 µm pore size through polycarbonate (PC) filters. Half of the filters were subjected to DNA-based quantification of *V. aestuarianus* (see below), and the other half of the filters were directly plated on the Zobell medium at 22°C for bacterial isolation (see below). Twenty millilitres of sediment and interstitial seawater was sampled into syringes, plated after agitation (10 min) and serial dilution up to 10^{-3} on the Zobell medium, and stored at −20°C for DNA extraction.

Quantification of *V. aestuarianus* DNA in oysters, seawater, and sediment: a pond survey

Total DNA was extracted from the haemolymph of live oysters, and filters were used to detect *V. aestuarianus* in seawater by means of the QIAamp Tissue Mini Kit (Qiagen) or NucleoSpin® Genomic DNA from Tissue Kit (Macherey-Nagel). Total DNA was extracted

from sediment with the PowerSoil DNA Isolation Kit (MoBio). DNA concentrations were measured using a spectrophotometer (Nanodrop, Thermo Fischer) before storage at –20°C. *V. aestuarianus* DNA quantification was carried out via a qPCR protocol (Saulnier et al., 2009) on Mx3000 and Mx3005 Thermocyclers (Agilent). Amplification reactions were carried out in duplicate, in a total volume of 20 µL. Each well contained 5 µL of genomic DNA (5 ng/µL), 10 µL of the Brillant III Ultra-Fast Master Mix (Agilent), 0.060 µL of each primer at 100 µM (DNAj-F 5′-GTATGAAATTTTAACTGACCCACAA, DNAj-R 5′-CAATTTCTTTCGAACAACCAC), 0.040 µL of DNAj probe 100 µM (5′-TGGTAGCGCAGACTTCGGCGAC), and 4.84 µL of distilled water. qPCR cycling conditions were as follows: 3 min at 95°C, followed by 40 cycles of amplification at 95°C for 10 s and 60°C for 20 s. By a similar protocol, total Vibrio DNA was estimated with 567F-680R primers (Thompson et al., 2004).

For experimental colonisation analyses, bacterial DNA concentrations were normalised in each sample to an oyster gene (elongation factor gene = Cg-EF, 5′-AGTCACCAAGGCTGCACAGAAAG-3′; 5′-TCCGACGTATTTCTTTGCGATGT-3′). Firstly, the copy numbers of genes, *dnaJ* and *Cg-EF*, were estimated by means of genomic DNA of *V. aestuarianus* and a plasmid containing the *C. gigas* EF gene, respectively (Segarra et al., 2014) as standards. Briefly, standards were prepared using the relation between the concentration of DNA and the theoretical copy number of a genome or plasmid, calculated on the basis of the DNA mass divided by molecular weight of the genome or of the plasmid. They were also validated by the limit dilution method, assuming that the dilution at which 1 replicate in 10 was positive corresponds to 1 copy. The absence of amplification (no Ct), or unquantifiable values (outside the standard range) were labelled as 1 copy and 10 copies

respectively. Finally, the ratio Log10 (absolute quantification of *V. aestuarianus dnaJ*) ÷ Log10 (absolute quantification of *C. gigas* EF) was calculated. The relative stability of our reference gene between individuals (Ct) was checked to ensure consistency of our normalised results (bacterial cells/haemocytes *vs* bacterial cells/ml of haemolymph).

Bacterial isolation, identification, and typing: a pond survey

Serially diluted samples (two sediment samples, 30 oyster haemolymph samples) and 5, 1, and 0.22 µm PC filters were plated on Zobell medium at 22°C with incubation for 48 h. From 135 to 460 colonies were boiled in distilled water and tested by qPCR as described above at each sampling time point to isolate as many *V. aestuarianus* clones as possible. All colonies positive for *dnaJ* amplification were re-streaked twice and stored at –80°C. For DNA sequencing, 5 µL of boiled DNA extract (10 min at 95°C) was added to a PCR mix (GoTaq G2, Promega) for lactate dehydrogenase (*ldh*) amplification (Goudenege et al., 2015). The PCR conditions were as follows: 5 min at 95°C, followed by 30 cycles of 30 s each of 95°C and 58°C and 1 min at 72°C, with a final step of 7 min at 72°C. Universal PCR analysis targeting the 16S rRNA gene (universal primers SAdir S17rev) was performed on negative results to confirm the absence of inhibitors in DNA extracts.

The partial *ldh* sequences were aligned with Muscle and phylogenetic trees were built in MEGA 6 (http://www.megasoftware.net/) by the maximum likelihood algorithm, with the GTR model, and reliability was assessed by the bootstrap method with 1000 replicates.

Experimental infection

Bacterial virulence estimation by injection

Bacteria were grown with constant agitation at 22°C for 24 h in the Zobell medium. One hundred microliters of diluted culture (10^2 cfu, estimated as OD_{600} of 0.002 and checked by plating on Zobell agar) was injected intramuscularly into anesthetised SPF oysters. After injection, the oysters were transferred to aquaria (10 oysters per aquarium of 2.5 L, triplicate aquaria per bacterial strain) containing 1 L of aerated UV-treated seawater at 22°C, kept under static conditions for 5 days.

Experimental immersion infection: immersion into seawater containing cultured bacteria

Seawater containing cultured bacteria was produced from a diluted concentrated bacterial suspension prepared as described above to reach a final concentration of ~5×10^8 bacterial cells/L. Sentinel oysters were immersed for 24 h in this water before 2-week following in fresh UV-treated seawater. This experiment was reproduced over time (14 times).

Experimental immersion infection: immersion into seawater containing freshly shed bacteria

The first step of this protocol corresponds to production of CSW, i.e. containing freshly shed bacteria. Source oysters (biparental families) were anaesthetised for 4 h at 22°C in a solution of magnesium chloride (50 g/L of freshwater) with aeration. The oysters were subsequently injected with 50 µL of one of the different bacterial suspensions into the adductor muscle. Afterwards, the animals (30 to 50 oysters for each condition) were placed into a tank containing 12 L of UV-treated seawater maintained under static conditions (one tank per

strain). A control has been realised in the same condition by injection of SASW. After 48 h, *V. aestuarianus* concentration in CSW was estimated by qPCR and was adjusted to 5×10^8 to 1×10^9 bacterial cells/L.

Sentinel animals (biparental families) were then exposed to this CSW or to seawater as control for 24 h under static and aerated conditions. Finally, after these 24 h of contamination, infected and control oysters were transferred into new tanks containing UV-treated seawater. This experiment was reproduced over time (17 times).

Sampling and mortality monitoring for histopathological analysis

During one experiment on freshly shed bacteria, eight tanks were designated for sampling (15 animals/tank, 4 tanks per condition, 3 conditions: oysters exposed to virulent 12/016 strain, oysters exposed to non-virulent 12/016 mutant and oysters not exposed to the bacteria). At different time points after infection (day 1 and day 4), haemolymph was sampled from the posterior adductor muscle sinus, by gentle aspiration with a 1 mL syringe equipped with a 22-G needle. Quality of samples was systematically checked by microscopic examination before use. Pieces of gills, of the mantle, adductor muscle, digestive gland, and labial palps were dissected and stored at –20°C. Finally, a half of the animal was fixed for histological analysis as described below.

The four other tanks were designated for mortality estimation. Examination of mortality was performed daily for 14 days. A dead oyster was defined as a moribund animal that was unable to close its valve after 5 min out of water. To confirm that the mortality is due to *V. aestuarianus*, haemolymph was sampled in moribund animals. From 100 µL, DNA was extracted, and *V. aestuarianus* DNA was quantified by *dnaJ* qPCR as described above. In

parallel, haemolymph samples were spread on the Zobell agar medium, and predominant bacteria were subjected to *dnaJ* qPCR to identify *V. aestuarianus* clones.

Oyster fixation and labelling for histopathological analysis:

Fixation

Oyster samples were preserved in Davidson's fixative (22% formaldehyde, 33% ethanol 95° proof, 12% glycerol, 33% of 0.22 µm–filtered seawater, and 10% acetic acid) for 48 h and then stored in ethanol before embedding in paraffin wax blocks. From each block of paraffin, consecutive 3- to 5-µm-thick sections were cut off and adhered to Superfrost (H&E) or Superfrost Plus (IHC) microscope slides.

Haematoxylin and eosin (H&E) staining

The *C. gigas* tissue slices were deparaffinised and stained with Histalim (Montpellier), by means of standard Harris' H&E stain according to ref. (Hayat, 1993), and mounted with the Eukitt Mounting Medium (O. Kindler GmbH). The slices were viewed under a microscope (Olympus BX50) coupled to a camera (Leïca DMC 2900). The slides were digitised by a Nanozoomer scanner (Hamamatsu) with a light background, via a 20× objective, without a Z stack. For lesions, beyond any abnormal characteristics, we mostly noticed the presence of necrosis, atrophy, and haemocyte infiltration into different tissues: gills, gonads, digestive glands, the mantle, muscle, kidney, and digestive tube. The lesions intensity in each organ was coded as follows: 0 (absence of a lesion); 1 (low), 2 (medium), and 3 (high). Moreover, for DD, lumen area of 30 DD/slide was measured in 14 oysters infected with WT, 12 oysters infected with the Δ*varS* strain, and in four non-infected oysters (NDP.view2, Hamamatsu.com).

Immunohistochemical analysis (IHC)

Immunostaining with Histalim (Montpellier) was performed on a Benchmark automate (Ventana-Roche). Briefly, oyster tissue slices were prepared and deparaffinised as described above, and next pre-hybridised in immunohistochemistry buffer (Tris/EDTA pH 8.9). The pre-hybridisation solution was replaced with fresh immunohistochemistry buffer containing a rabbit polyclonal anti-[Va] primary antibody (gift from JL Nicolas, LPI Ifremer Plouzané) for 1 h at a 1:20 000 dilution. The specificity of this antibody was firstly tested by western blotting and dot blotting, and the conditions were optimised. After washing and incubation with a secondary antibody, analysis with the Ultraview Red AP Kit (Roche Diagnostics) was performed. The slices were viewed under a microscope (Olympus BX50) coupled to a camera (Leïca DMC 2900). The slides were digitised by the Nanozoomer scanner (Hamamatsu) against a light background, via a 20× objective, without a Z stack. A negative control was set up by omission of the primary antibody.

Statistical analyses

These analyses were performed in the XLSTAT software (Version 2011.4.02). To validate the ecological preferences of *V. aestuarianus* and to study the distribution in oyster haemolymph and seawater, the Kruskal–Wallis and Fisher's exact tests and odds ratio analysis were performed. To compare the DD lumen between the control and infected animals sampled at different time points, lumen perimeters were compared by the Mann–Whitney test.

Figure legends

Figure 1 : Oyster disease dynamic on field. The 7th October 2014 (week 0, x axis) SPF oysters (recipient, n=1500) were exposed to natural seawater in a pond containing *V. aestuarianus*-infected oysters (donor, n=2000). During 15 weeks (x axis) mortality rates of the recipients were recorded weekly (red line, %, y axis). At each time point, temperature (blue dotted line, °C, y axis) and salinity (green dotted line, g/L, y axis) were measured.
At five dates (weeks 0, 3, 6, 9, 15), living animals (n=20) were returned to the laboratory to allow development of the disease in tanks at 25°C and their cumulative mortalities after 7 days are indicated by read bars (%, y axis).

Figure 2: Quantification of *V. aestuarianus* and total *Vibrio* DNA by qPCR (y axis) in sediment (grey), seawater fractions (red, zooplankton; yellow, large phytoplankton and organic particles; green, smaller organic particles; blue, free-living bacterial cells) and oyster hemolymph (purple, average on 30 individuals) sampled at the indicated week (x axis) during the field's survey (Figure 1). Quantifications are expressed as genome units per g in sediment, per L in seawater and per mL in oyster hemolymph,

Figure 3: Oyster disease dynamic in mesocosm. Oysters (n=30) were transiently immersed (for 24h) in contaminated seawater containing 5.10^8 cfu/L of V. aestuarianus WT strain (red line), and its Δ*varS* derivative (green line) or UV treated seawater (blue line), washed and transferred to new tank containing UV treated water. Mortalities were recorded daily for 14 days (x axis) and cumulative mortality rates are indicated in % (y axis). The four different infection phases are also indicated.

Figure 4: Infection dynamics of *V. aestuarianus* in animals infected with a highly pathogenic strain (WT) and with a non-pathogenic strain (Δ*varS*). *V. aestuarianus* DNA amounts were quantified by qPCR in hemolymph (A), mantle (B), gills (C), muscle (D), digestive gland (E) and palps (F). Scattergrams show the bacterial DNA copy number/cell. The change in bacterial DNA concentration for each sample is depicted on the y axis as a data point with the Log 10 DnaJ (*V. aestuarianus* gene) normalized by Log10 Cg-EF (oyster elongation factor Cg-EF). X axis represents the post infection sampling (d1: day1 post infection; d4: day4 post infection and mo.: moribund animals). Red line corresponds to the median. 15 animals were analyzed per time and per condition, except for controls for which 5 animals were sampled.

Figure 5: Bacterial colonization observed in individuals infected with virulent *V. aestuarianus* strain. Immunohistochemistry allowed us to identify 3 steps of infection: early (B, day 1, 14 animals), intermediate (C-D, day 4, 15 animals) and moribund state (E, 4 animals). Two groups of distribution were noticed on day 4 and defined as intermediate group a (C, 8 animals, labelling in gills only), and intermediate group b (D, 7 animals, labelling in all tissues). Different tissues were studied: gills (G), digestive gland (DG), muscle (Mu), mantle (Mtl) and labial palps (Plp) for controls and infected animals. With ultra view red kit, a pink/violet labeling of bacterial-like cells was observed in different tissues. All scale bars = 100 µm.

Figure 6: Histological sections from adult oysters stained by hematoxylin-eosin showing 3 types of tissue lesions indicated by arrows. Upper sections (A-B) correspond to mantle, middle sections (C-D) to digestive diverticula, and lower sections (E-H) to connective tissues

and vessels with hemocytes. Sections A, C and E correspond to controls (8 animals studied) and sections B, D, F-H to highly infected animals (4 animals studied). The different lesions noticed are: (1) lysis of the subepithelial connective tissue in mantle (section B), (2) atrophy of digestive diverticula with an increase in diverticulum lumen (section D), and (3) hemocyte infiltrations (section F), lysis and agglutination (section G in sinus and section H in artera). *: significant increase in lumen area. Scale bar = 100 µm (A-F) or 50 µm (G-H).

Supplementary figures and tables

Supplementary Figure 1: Quantification of *V. aestuarianus* in oyster hemolymph individuals by qPCR (genome units per mL, 30 live animals, y axis) sampled at the indicated week (x axis) during the field's survey (Figure 1). Means are represented by red crosses and median by red lines.

Supplementary Figure 2: Oyster experimental infection by immersion into *V. aestuarianus* contaminated waters. Oysters (n=30) were transiently immersed into waters containing 5.10^8 cfu/L of *V. aestuarianus* cultured overnight in a rich media, Zobell (14 distinct experiments, grey bars, x axis) or freshly shed from injected oysters (17 distinct experiments, black bars, x axis). Cumulative mortalities rate obtained after 14 days are given (y axis).

Supplementary Table1: Quantification of total vibrios (Tot) and *V. aestuarianus* (*V. a*) genome copy number in seawater fractions (B/L), in sediment (B/mg) and oyster hemolymph (B/ml) sampled at week 0, 3, 6, 9 and 15 during the field's survey (Figure 1). Percentage of *V. aestuarianus* out of total vibrios (ratio) is also indicated. nd: not determined, neg: no detection

Supplementary Table 2: Number of bacteria isolated at week 0, 3, 6, 9 and 15 in seawater fractions and sediment, or oyster hemolymph during the field's survey (Figure 1) and screened to identify *V. aestuarianus* strains.

Supplementary Table 3: Number of individuals for which bacterial labeling was observed in gills, digestive gland, mantle, labial palps or muscle. Control oysters (8 individuals) and oysters infected with *V. aestuarianus* WT strain (33 individuals) or Δ*varS* derivative (20 individuals) were analyzed. Immunohistochemistry was performed using anti-aestu polyclonal antibody and ultra view red kit. For individuals infected with WT and sampled on day 4, two groups of individuals were defined based on the intensity and the repartition of the labeling (Group a and b)

Supplementary Table 4: Number of individuals for which tissue lesions were observed in mantle, digestive diverticula or hemolymphatic compartment. Oysters infected with *V. aestuarianus* WT strain (33 individuals) or Δ*varS* derivative (20 individuals) were analyzed. Intensity of lesions was coded, considering the frequency of observations on 5 random fields of view for each tissue: low = up to 5 observations, medium = between 5 to 15 observations, high = more than 15 observations. For digestive diverticula atrophy, lumen areas were measured (on 30 diverticula, on 3 random fields) with NDP.view2 and compared by t-test. Observations were realized on hematoxylin eosin stained sections. For individuals infected with WT and sampled on day 4, two groups of individuals were defined based on the labelling observed by immunohistochemistry (table 4, Group a and b).

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The authors declare that they have no conflict of interest.

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Figure 1

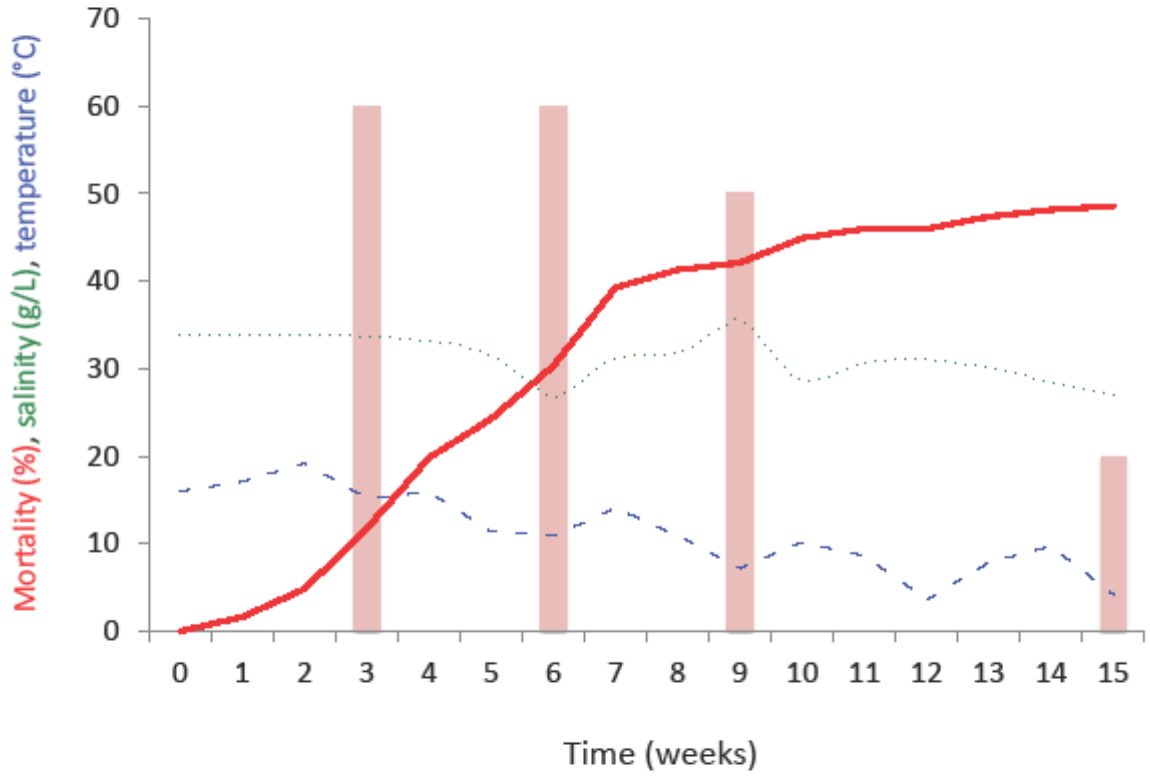
Mortality (%), salinity (g/L), temperature (°C)
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time (weeks)

Figure 2

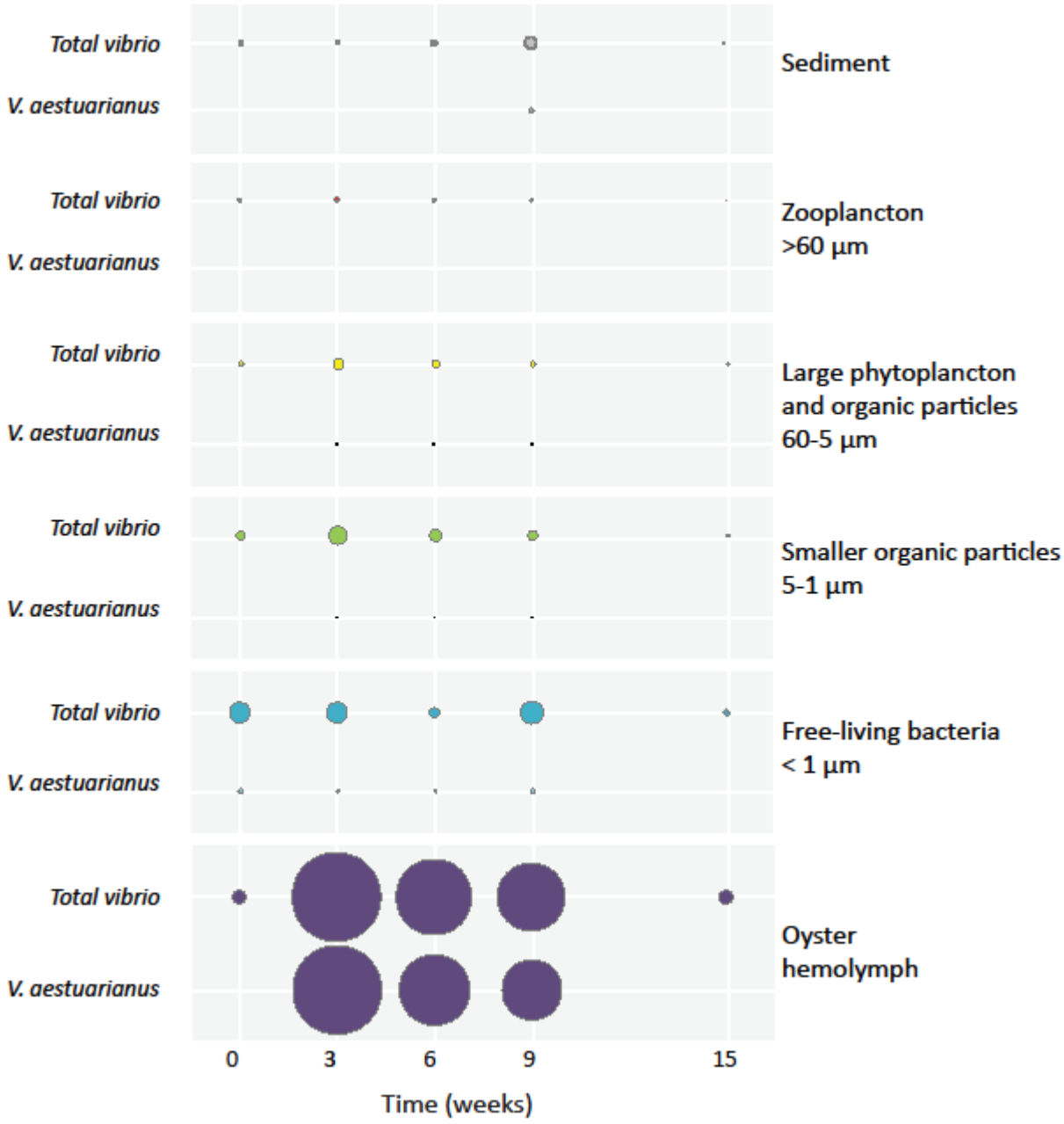
Total vibrio
V. aestuarianus
Sediment
Total vibrio
V. aestuarianus
Zooplancton
>60 µm
Total vibrio
V. aestuarianus
Large phytoplancton
and organic particles
60-5 µm
Total vibrio
V. aestuarianus
Smaller organic particles
5-1 µm
Total vibrio
V. aestuarianus
Free-living bacteria
< 1 µm
Total vibrio
V. aestuarianus
Oyster
hemolymph
0
3
6
9
15
Time (weeks)

Figure 3

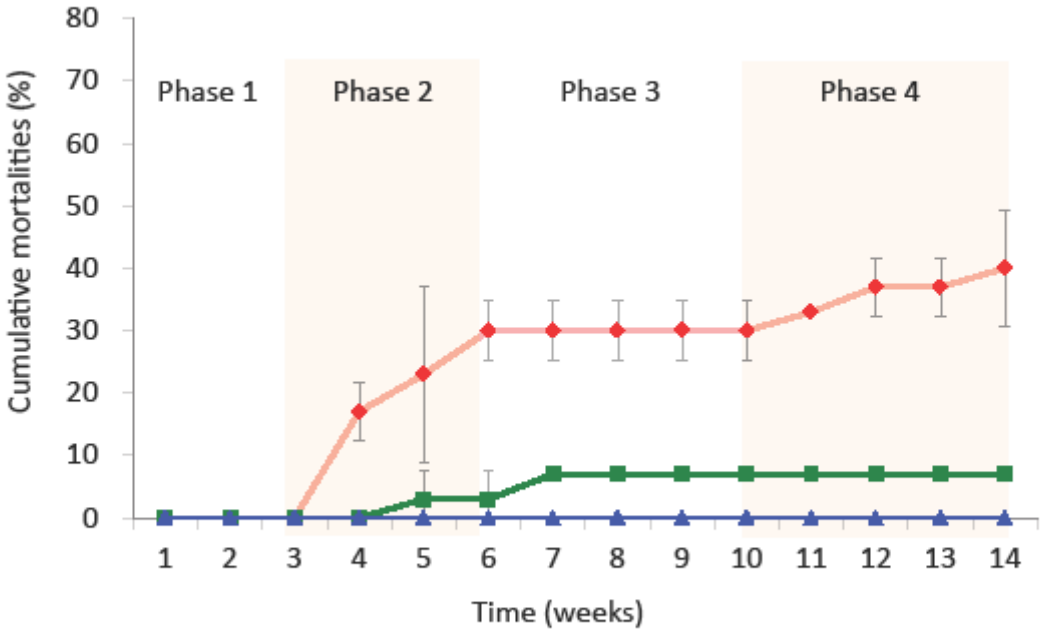
Cumulative mortalities (%)
80
70
60
50
40
30
20
10
0
Phase 1
Phase 2
Phase 3
Phase 4
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Time (weeks)

Figure 4

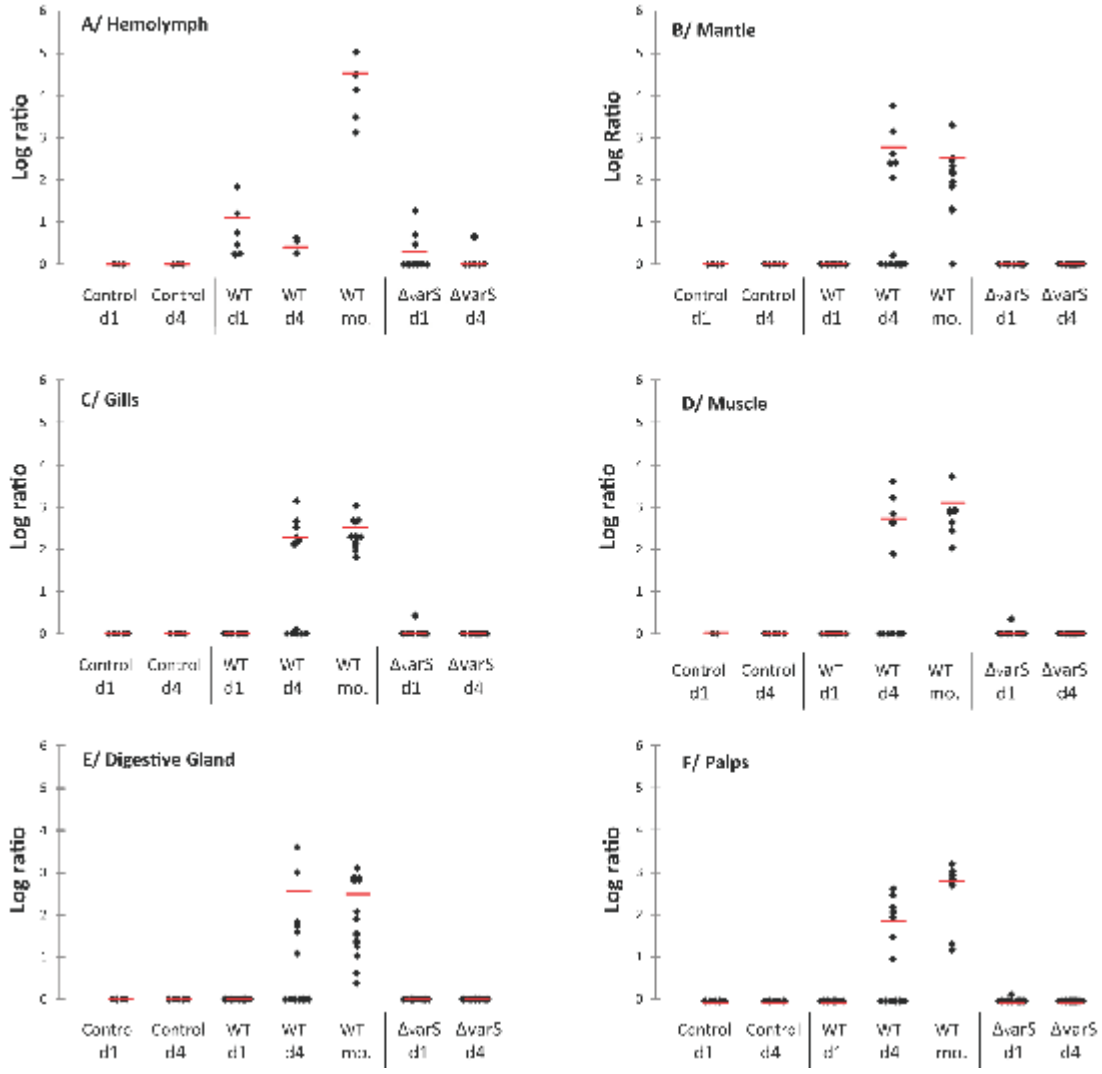
A/ Hemolymph
B/ Mantle
C/ Gills
D/ Muscle
E/ Digestive Gland
F/ Palps
Log ratio
Log Ratio
Control d1
Control d4
WT d1
WT d4
WT mo.
ΔvarS d1
ΔvarS d4

Figure 5

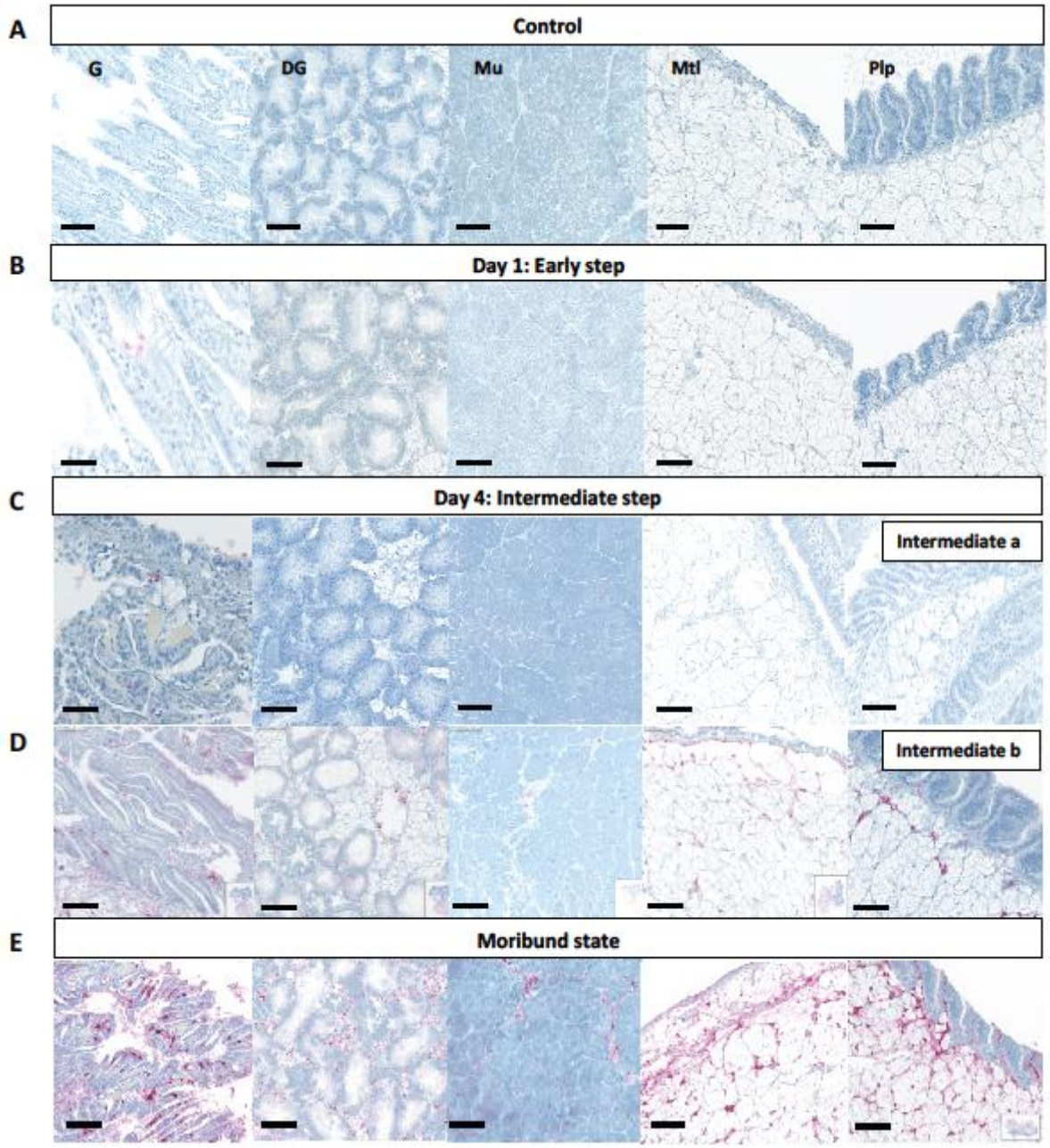
A
Control
G
DG
Mu
Mtl
Plp
B
Day 1: Early step
C
Day 4: Intermediate step
Intermediate a
D
Intermediate b
E
Moribund state

Figure 6